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THE EFFECTS OF LOW ENVIRONMENTAL TEMPERATURE UPON METABOLISM*

I. TECHNIC AND RESPIRATORY QUOTIENT

By Raymond W. Swift†
(From the Department of Vital Economics, University of Rochester, Rochester, N. Y.)

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ALTHOUGH it has been known since the time of Lavoisier that a low environmental temperature increases the metabolism in man, there are not at present concordant opinions in respect to the various aspects of this increase. In two cases out of three Lusk (1) found that one of the characteristic effects of cold was a relative increase in carbohydrate combustion. Rubner (2) also observed an increase in the respiratory quotient as well as in oxygen consumption as a result of a cold bath. Loewy (3), however, concluded as a result of an extensive study that the changes in respiratory quotient resulting from exposure to cold were due entirely to under- and over-ventilation.

This paper reports the findings pertaining to the foodstuffs oxidized during exposure to cold.

Experimental

Twenty-one subjects, men and women, were exposed in a basal condition to an environmental temperature of about 2° C. and 80 per cent relative humidity. Most of the subjects were medical students and all were in normal health with the possible exception of A.D. who had been previously treated for exophthalmic goitre. Thirty-seven basal metabolism measurements are reported along with 112 observations taken during exposure to the cold environment. Two separate tests on different days were made with 15 of the subjects.

The metabolism was measured by the method of Tissot (4), essentially as modified and described by Boothby and Sandiford (5). The basal metab-

* Submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy, Department of Vital Economics, University of Rochester, Rochester, N. Y.

† On leave of absence from the Institute of Animal Nutrition, Pennsylvania State College.

olism was determined at room temperature (23–25° C.), all conditions pertaining to the proper measure of basal metabolism as outlined by Lusk (6) being rigidly observed.

An army cot, the canvas of which had been replaced by wire netting, was used throughout. This proved to be perfectly comfortable to the subject and as neither blanket nor mattress was used, this arrangement

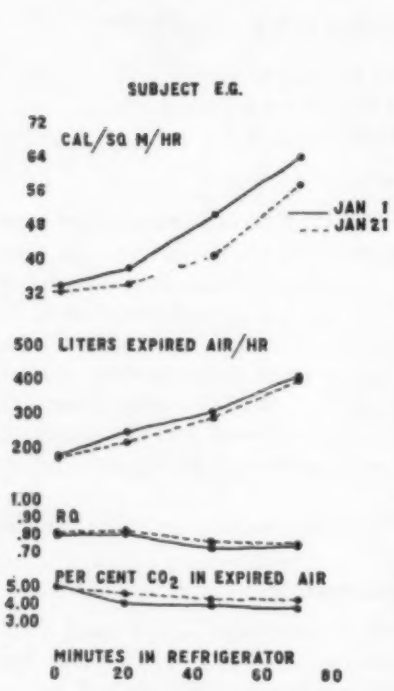

CHART 1

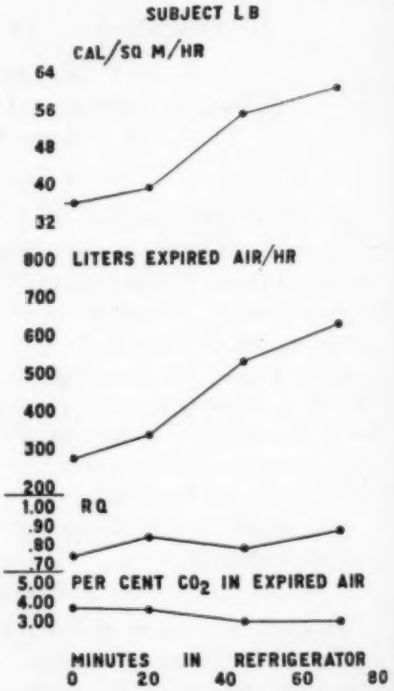

CHART 2

served to expose the whole body surface to the surrounding air. The clothing worn by all the subjects at all times was as nearly identical as possible, consisting in the case of the male subjects of shoes and stockings, light underwear, trousers, shirt, vest, and service coat. The female subjects were likewise clothed in the ordinary clothing worn in the laboratory. Though there undoubtedly may have been a slight difference in the protection offered by the clothing of different individuals, each individual wore exactly the same clothing in duplicate tests.

After the determination of the basal metabolism, the subject was moved into the refrigerator for a period of 75 minutes. No blanket, mattress, nor extra clothing was used. During the 75-minute refrigerator period, three ten-minute samples of expired air were collected for analysis.[1] These represented the metabolism after exposure to the cold for 20, 45, and 70 minutes respectively. An additional period was run with two of the subjects, making the total time spent in the refrigerator 100 minutes. The subjects inhaled outdoor air in all of the refrigerator periods and in most of the basal metabolism determinations, though in some cases room air was used. As pointed out by Boothby and Sandiford (5), this has no appreciable effect upon the results. The subject was connected to the apparatus by means of a mouthpiece throughout the entire refrigerator period and immediately after the collection periods was allowed to change position slightly to avoid becoming cramped.

Technical Error

Analysis of the expired air was made with a Haldane apparatus as modified by du Vigneaud (7). In the use of this excellent type of apparatus Carpenter (8) prescribes an allowable variation between duplicate determinations of .003 per cent CO_2 and .005 per cent oxygen. Though the agreement between duplicates in this work was not always quite so close as this, the differences were never so great as .010 per cent and usually considerably less, especially in the case of CO_2. Even if the CO_2 and oxygen determinations are in error by .01 per cent and in such a direction as to be additive in their effects on the respiratory quotient (which as explained by Carpenter (9) is not likely to be the case) the respiratory quotient would be changed by only .003. In so far as the air analysis is concerned then, the respiratory quotient cannot be in error by more than .003. It should be borne in mind, of course, that this is true only for the analysis of expired air. In respiration chambers where the composition of the outcoming air is more nearly like atmospheric air, the accuracy of the determination of the respiratory quotient decreases accordingly. Analyses of outdoor air at intervals verified the correctness of results.

Other small errors involved with the measurement of the temperature and the volume of the expired air which have been well discussed by Boothby and Sandiford (5) cannot, even if additive, amount to 1 per cent in the computed heat production. These latter errors do not, of course, have any effect whatsoever on the respiratory quotient since it is deter-

[1] A few samples were taken over a somewhat shorter interval usually because of the limiting factor of the spirometer volume.

mined solely by the composition of the air. Due to the small experimental error, small differences obtained with the same or different individuals may safely be attributed to physiological variation.

This open-circuit method of indirect calorimetry presents several advantages over the closed circuit type of apparatus which have been well discussed by Carpenter (9), Murlin (10), Boothby and Sandiford (11) and Du Bois (12), and will not be further mentioned here.

Short time experiments involving a critical interpretation of the respiratory quotient must include a careful consideration of "*Auspumpung*" and the compensatory process which follows it. This important point will now be considered in connection with the results obtained.

Auspumpung and the Significance of the Respiratory Quotient

A careful consideration of the interpretation and significance of the respiratory quotient has been given by Richardson (13) and by Cathcart and Markowitz (14). They emphasize the point that the respiratory quotient represents the algebraic sum of many factors and reactions. Boothby *et al.* (15) state that the above authors in expressing caution in the literal and direct interpretation of rises in respiratory quotients that do not go above unity "have voiced the doubts of all careful workers in the subject."

Auspumpung is a German word which refers to the "blowing off" of more CO_2 than is actually produced during the time of measurement and which results from hyperventilation. A consideration of this extra CO_2 elimination has been well emphasized by Murlin *et al.* (16), and that it may be a complicating factor in all metabolic studies is mentioned by Colwell and Bright (17). An increase in effective respiration may be due to increase in rate or in depth or in both and may be the result of a variety of causes. Lusk (6) quotes some unpublished work by Sturgis showing the influence of voluntary deep breathing for short periods upon the respiratory quotient during which time the respiratory *rate* remained unchanged. In the first period (9 minutes) in which the subject breathed normally, the respiratory quotient was 0.80. In the second period (9 minutes) in which the breathing was much deeper though no faster, a respiratory quotient of 1.43 was obtained. During the next period, which immediately followed, and in which the subject was instructed to ignore his breathing, a respiratory quotient of 0.53 was obtained. Weiss (18) has reported essentially the same results, obtaining a respiratory quotient of 1.34 during a very short period of forced respiration and one of 0.66 in the following recovery period. An important point is the very short time necessary to eliminate a relatively large amount of CO_2 and this point is significant in the evalua-

tion of the few high respiratory quotients observed in this work during a few refrigerator periods. According to Bornstein and Gartzen (19) overventilation by voluntary effort in human subjects is limited to 50 minutes as regards the elimination of extra CO_2. By voluntary fast breathing, Coleman and Du Bois (20) obtained a change in the apparent respiratory quotient from 0.77 to 1.10 in 15 minutes. Kilborn (21), using artificial respiration in his work on decerebrate cats, discounts the high respiratory quotients obtained largely because of the washing out of CO_2 from the blood.

In discussing this blowing off of CO_2 Du Bois (12) says that, "Next to leaky apparatus, it has caused more trouble than any other factor. It has filled the literature with erroneous data and faulty conclusions most difficult to eradicate"—also that it "has played havoc with countless experiments on the basal metabolism and will continue to do so for years to come." In appreciation of the truth of the remarks of Du Bois, the results of this work in this respect will be fully considered and discussed.

Unfamiliarity with the apparatus may cause the subject to breathe abnormally. But that this was not the case in the results here reported is indicated by the fact that the respiratory quotient of the basal metabolism, obtained before the subject was exposed to cold, is in no case questionably high. Furthermore, most of the subjects had served as subjects before. It will be noted also that in most cases the rise in respiratory quotient occurred toward the end of the refrigerator period, after the subject had been connected to the apparatus for a considerable time.

With but very few exceptions the percentage of CO_2 in the expired air decreased in successive refrigerator periods in a perfectly regular manner, indicating a continuous and slightly increasing hyperventilation. In as much as the respiratory quotient, as measured, often remained constant, in spite of some degree of hyperventilation, it seems quite possible that the true respiratory quotient tends to drop during exposure to cold. Since only 3 ten-minute air samples were taken during the 75-minute exposure to the cold, the extra CO_2 eliminated as a result of hyperventilation was quantitatively measured only in so far as the extra elimination occurred during the time the samples were being taken. If it occurred *only before* a sample was taken, so that a new and lower alveolar CO_2 tension had been established which was then maintained throughout the collection period, then the evidence that *Auspumpung* had taken place would consist of the observation of a large volume of expired air in proportion to the oxygen consumed, and a decreased percentage of CO_2 in the expired air. The respiratory quotient, if no change was taking place as to foodstuffs oxidized,

would remain unchanged. As pointed out by Starling (22), the composition of the expired air represents the composition of the alveolar air diluted with an appreciable amount of air which remains in the "dead space." Bronchial constriction, as a reflex effect of cold, might lessen this space and thus affect the composition of the expired air. Such constriction, however, would tend to raise the percentage of CO_2 in the expired air whereas in all cases the percentage of CO_2 decreased and the decrease was accompanied by a large increase in ventilation.

TABLE I

AGE, WEIGHT, HEIGHT, BODY SURFACE, AND SEX OF SUBJECTS

Subject	Sex	Age	Weight kg.	Height cm.	Surface area* sq. m.
A.A.	Female	21	64.8	166.0	1.72
E.G.	"	25	60.9	163.0	1.65
A.B.M.	"	33	58.4	164.0	1.63
M.M.	"	40	41.9	143.0	1.28
R.E.S.	Male	25	65.2	176.5	1.80
S.P.	"	22	73.8	184.0	1.96
W.D.	"	23	75.2	179.5	1.94
A.P.	"	26	69.3	171.5	1.81
J.M.	"	57	93.8	184.2	2.17
J.R.	"	15	73.6	174.0	1.88
A.D.	Female	21	51.4	164.0	1.55
E.H.	"	31	59.0	157.5	1.59
L.B.	Male	25	72.0	182.0	1.92
V.S.	"	23	75.2	179.5	1.94
T.S.	"	22	69.5	171.0	1.81
C.H.	"	27	113.4	179.0	2.28
R.D.	"	26	88.5	177.5	2.06
E.L.G.	"	25	89.3	178.0	2.09
L.M.	"	24	82.0	179.0	2.01
H.H.	"	25	78.9	168.5	1.89
E.B.	Female	26	51.4	151.0	1.46

* Du Bois height-weight formula.

When there is no change in the oxygen consumption, the process of eliminating extra CO_2 from the blood by hyperventilation must diminish the alveolar CO_2 tension and, therefore, the percentage of CO_2 in the expired air. Hence, any change in the CO_2 content of the expired air is an indication of the CO_2 changes in the blood (Finney *et al.* (23)). The volume of expired air during hyperventilation is large as compared with the oxygen consumption and a knowledge of the volume of the expired air furnished

TABLE II

METABOLISM OF SUBJECTS EXPOSED TO A LOW ENVIRONMENTAL TEMPERATURE

Subject	Period	Refrigerator		Liters expired air per hr.	R.Q.	% CO_2 in expired air	Rectal temp. after basal and after exposure to cold. °C
		Temp. °C	% relative humidity				
A.A.*	Basal			232.2	.805	4.940	37.0
	1	1.3	81	377.6	.801	5.018	
	2			562.1	.827	4.760	
	3	3.3	68	574.6	.810	4.530	36.8
E.G.	Basal			191.5	.825	5.216	—
	1	1.1	81	258.5	.831	4.316	
	2			322.5	.750	4.239	
	3	5.0	58	423.5	.762	4.119	—
E.G.	Basal			185.4	.837	5.177	36.80
	1	0.6	80	232.8	.849	4.880	
	2			302.7	.790	4.605	
	3	1.9	72	422.1	.783	4.645	36.50
A.B.M.§	Basal			259.7	.816	4.260	37.05
	1	−0.6	78	324.2	.810	4.175	
	2			375.3	.794	3.985	
	3	2.6	79	451.7	.790	4.012	37.00
M.M.*	Basal			144.3	.819	4.979	36.85
	1	−0.6	84	181.6	.815	4.842	
	2			245.7	.803	4.642	
	3	1.8	79	353.7	.802	4.329	36.70
R.E.S.*	Basal			236.4	.811	4.581	36.60
	1	0.7	80	279.6	.836	4.730	
	2			435.2	.822	4.342	
	3	4.4	71	462.9	.793	4.507	36.40
S.P.	Basal			285.5	.792	4.714	36.20
	1	0.6	80	326.2	.776	4.548	
	2			350.3	.778	4.391	
	3	2.8	74	410.7	.758	4.430	36.05
W.D.*	Basal			289.7	.787	4.502	36.65
	1	−0.2	84	313.3	.812	4.563	
	2			347.1	.835	4.343	
	3	3.6	72	626.4	.872	3.829	36.40
A.P.*	Basal			256.7	.854	4.558	36.55
	1	−0.3	79	357.7	.863	4.272	
	2			427.6	.838	4.185	
	3	4.2	72	514.9	.810	4.377	36.50
J.M.*	Basal			299.6	.829	4.134	36.95
	1	0.4	79	484.2	.902	3.583	
	2			625.0	.869	3.320	
	3	3.7	71	764.0	.873	3.010	36.70

* Average of two tests on different days.

§ Average of 3 tests.

TABLE II (cont'd)

Subject	Period	Refrigerator		Liters expired air per hr.	R.Q.	% CO_2 in expired air	Rectal temp. after basal and after exposure to cold. °C
		Temp. °C	% relative humidity				
J.R.*	Basal			332.2	.801	4.342	36.70
	1	0.2	80	387.4	.802	4.310	
	2			477.8	.822	4.021	
	3	3.8	67	771.0	.870	3.534	36.50
A.D.*	Basal			249.6	.788	4.260	36.65
	1	−0.2	81	370.8	.773	4.122	
	2			411.1	.764	4.129	
	3	1.5	79	507.7	.771	4.098	36.75
E.H.*	Basal			198.4	.785	4.518	36.45
	1	0.6	80	354.9	.790	4.404	
	2			383.4	.794	4.167	
	3	3.2	75	377.7	.792	4.032	36.35
L.B.	Basal			291.6	.792	4.018	36.85
	1	1.2	81	351.3	.868	3.961	
	2			543.2	.809	3.367	
	3	4.4	63	640.5	.897	3.389	36.70
V.S.	Basal			244.7	.843	4.561	36.70
	1	−1.0	76	568.0	.879	3.783	
	2			350.2	.819	4.401	
	3	1.9	81	483.8	.800	4.527	36.70
T.S.	Basal			225.7	.816	4.776	36.70
	1	0.1	79	264.8	.860	4.507	
	2			448.1	.988	4.160	
	3	2.8	75	818.9	1.027	3.474	36.55
C.H.	Basal			354.0	.789	4.147	36.60
	1	0.0	79	362.2	.822	4.189	
	2			339.1	.790	4.114	
	3	3.1	87	363.3	.788	3.965	36.90
R.D.*	Basal			288.1	.837	4.735	36.95
	1	0.8	81	306.8	.879	4.688	
	2			282.2	.799	4.644	
	3	4.6	69	264.8	.750	4.774	36.95
E.L.G.*	Basal			256.5	.766	4.826	36.80
	1	−0.1	79	265.7	.796	4.764	
	2			266.8	.784	4.742	
	3	2.8	80	294.1	.797	4.531	36.75
L.M.*	Basal			276.8	.819	4.517	36.80
	1	0.4	80	300.7	.838	4.506	
	2			294.7	.805	4.424	
	3	3.8	71	323.6	.782	4.544	36.50
H.H.*	Basal			211.1	.823	4.932	36.65
	1	0.7	81	268.5	.900	4.584	
	2			288.1	.858	4.487	
	3	3.5	69	351.4	.823	4.382	36.40
E.B.	Basal			163.0	.752	5.006	36.55
	1	0.6	80	225.0	.785	4.732	
	2			223.9	.800	4.728	
	3	2.6	78	309.6	.777	4.680	36.05

* Average of two tests on different days.

another criterion in evaluating respiratory quotients in metabolism tests where *Auspumpung* was suspected. Hyperventilation also results in a higher percentage of oxygen in the expired air. Observations on the rate and minute volume of respiration are directly indicative. When positive findings are made in regard to all the above factors, together with a questionably high respiratory quotient, it is safe to assume that the rise in respiratory quotient is merely superficial.

Lack of space prohibits the presentation of all the individual data which are outlined in Table II. In most cases the respiratory quotients obtained during exposure to cold were scarcely different from that of the basal. This constancy was by no means confined to those subjects who showed no marked increase in metabolism. It is true also in many cases which show a very pronounced increase in heat production and in the case of A.A., who showed the greatest increase of any subject (150 per cent), the respiratory quotient was constant indeed. Shivering by this subject was very vigorous and practically continuous. A pneumograph was not in use at the time of these tests but with only one other case was shivering so marked as to cause the spirometer bell to quiver with each expiration of the subject. Thus shivering does not necessarily produce *Auspumpung* but may be accompanied by it. The CO_2 content of the expired air decreased progressively with this subject as with others but no marked drop is observable between any two consecutive periods.

As mentioned above, the results of hyperventilation may be manifest in a very few minutes. An example of this is found in the case of the first test with E.G. (Chart 1). The percentage of CO_2 in the expired air during the basal was 5.216 while during the first refrigerator period it was only 4.316 per cent, this latter value decreasing but slightly during the subsequent refrigerator period. The first air sample in all cases was collected during a 10-minute interval which began 15 minutes after the subject entered the refrigerator. In this instance considerable *Auspumpung* apparently occurred during the 15 minutes *before* the sample was taken, as indicated by the pronounced and sustained drop in the percentage of CO_2 which is not accompanied by an increase in the respiratory quotient. All the observations pertaining to *Auspumpung* mentioned above indicate that in the second test with this subject the same thing occurred to a less degree and somewhat later, so that the blowing off of CO_2 was still taking place to some extent during the first collection period. This is indicated by the rise in respiratory quotient, the drop in the percentage of CO_2 in the expired air, and in an increase of 25.5 per cent in the volume of expired air over the basal, with no increase in oxygen consumption. The change in re-

spiratory quotient from 0.837 to 0.849, though appreciable, certainly does not indicate increased combustion of carbohydrate. An exhaustive consideration of all instances showing a rise in respiratory quotient leads to the inevitable conclusion that the rise results from the blowing off of CO_2.

As a further check on the superficiality of the few high respiratory quotients obtained, a "recovery period" was run on subject L.B. (Chart 2). The apparent respiratory quotient which was 0.897 during the last refrigerator period dropped to 0.730. This 10-minute period at room temperature, was taken 20 to 30 minutes after exit from the cold. A sample taken sooner would undoubtedly have given an even greater drop in the respiratory quotient.

The respiratory quotients obtained with subject V.S. are in keeping with the very exceptional values obtained as to the irregularity of the oxygen absorption, ventilation, and CO_2 content of the expired air.

Subject T.S. showed a progressive hyperventilation throughout and the apparent respiratory quotient rose to more than unity in the last refrigerator period. A regular decrease in the percentage of CO_2 in the expired air with an increased respiratory volume greatly in excess of the increased oxygen absorption is apparent in these periods.

An inspection of data obtained following the ingestion of 300 grams of meat, a preliminary report of which is given elsewhere (24), shows that the percentage increase in volume of expired air never exceeds the percentage increase in oxygen consumption. Exposure to cold, on the other hand, especially in periods where a rise in respiratory quotient is observed, results in a very much larger increase in expired air volume than in oxygen absorption, and the CO_2 content of the expired air invariably decreases. The same apparatus was used in both cases.

A special experiment was performed with subject T.S. By use of two spirometers all of the expired air was collected over the entire observation period of 1 hour and 34 minutes. The details of the experiment were as follows: The two spirometers were swept out with expired air from the subject who, in a basal condition, was lying on a cot in a room at 23° C. By throwing the valve on one of the spirometers the air was deflected from one into the other. After a 12-minute collection period, and as soon as the valve had been turned, the room was cooled by opening windows. A sample was taken from one spirometer while the other was being filled. After the sample was taken, the spirometer was completely emptied to be ready for the next interval. There was of course a slight contamination from the small amount of air left in the spirometer when it became mixed with the sample next collected in it. The residual air was very small in amount,

however, and the very small error involved tends primarily to make the example of *Auspumpung* less pronounced rather than to exaggerate the situation. The following table shows beyond any doubt that this subject reacted in this experiment as he did in the refrigerator and that the pronounced rise in respiratory quotient is entirely superficial.

DATA OBTAINED DURING EXPOSURE TO COLD AND DURING THE RECOVERY PERIOD

Time in minutes		R.Q.	Liters expired air per hour		% CO_2 in expired air
			Total	Per liter O_2 absorbed	
Basal	12.5	.841	221.5	16.7	5.072
Exposed to cold	11.5	.863	263.9	16.8	5.185
	16.3	.861	224.5	16.9	5.144
	8.4*	1.003	343.1	20.9	4.835
	7.2	.749	295.0	15.3	4.939
	7.7	.841	237.0	16.7	5.080
	12.1	.794	200.8	15.0	5.329
	10.3	.807	229.2	15.1	5.381
	8.0	.800	203.3	14.9	5.418
Average		.840			

* Vigorous shivering.

Vigorous shivering occurred during the third period following the basal. At the end of this period the windows were closed and the subject covered with blankets. The room temperature had dropped to about 10° C. with rapid movement of air currents. If the subject could have been warmed up more rapidly, a greater drop in respiratory quotient would have been apparent as well as a quicker return to the basal value.

The demonstration is so clean cut as to necessitate but little comment. The basal respiratory quotient of 0.841 is to be compared with the average of all the periods which is 0.840. The "weighted" or true average of all happens to be exactly 0.841. Exposure to cold accompanied by vigorous shivering, therefore, has no effect whatsoever on the true respiratory quotient.

A noteworthy observation is that the percentage of CO_2 during the period giving a respiratory quotient of 1.003 dropped only to 4.835 per cent from 5.144 per cent—its value in the preceding period. The short period of 8 minutes was thus long enough to give a high respiratory quotient but too short to lower the alveolar CO_2 tension markedly. A fact of significance is that the volume of air expired per liter of oxygen absorbed is greatest where the respiratory quotient is highest. Thus the instances

which show a definite rise in respiratory quotient after exposure to cold have all the earmarks of extra CO_2 elimination.

Summary

Thirty-seven basal metabolism measurements on humans are reported along with 112 observations taken during exposure to cold. A critical study of the apparent respiratory quotients obtained, clearly indicates that exposure to cold does not favor any preferential oxidation of carbohydrate. Depletion of the glycogen stores is, therefore, merely associated with, and proportional to, the increased total metabolism.

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THE EFFECTS OF LOW ENVIRONMENTAL TEMPERATURE UPON METABOLISM*

II. THE INFLUENCE OF SHIVERING, SUBCUTANEOUS FAT, AND SKIN TEMPERATURE ON HEAT PRODUCTION

By RAYMOND W. SWIFT

(From the Department of Vital Economics, University of Rochester, Rochester, N. Y.)

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IN MEASURING the metabolism of a fasting man exposed to cold, Voit (1) found an increase of 34.5 per cent in the CO_2 production which occurred in the absence of shivering. The work of Rubner and Lewaschew (2) supported Voit's observations. Campbell *et al.* (3) determined the metabolism of subjects exposed to the rather cold open air and concluded that shivering was not necessary to raise the metabolism. Hill and Campbell (4) in similar experiments found increases of about 43 per cent during the exposure of normal and pathological subjects to cold outdoor air. The work of Cannon *et al.* (5) with humans indicates that a considerable increase in metabolism in response to a "heat debt" may ensue without any accompanying shivering.

Loewy (6), however, concluded that increases in metabolism took place only when accompanied by shivering. This latter opinion was also expressed by Johansson (7) and by Sjöström (8) both of whom measured the CO_2 output when the subject was naked and exposed to cold. Benedict (9) is inclined to agree with them, and Morgulis (10) concludes, from results obtained with dogs, that metabolism increases on exposure to cold only because of a heightened state of muscle tension.

The primary purposes of the work reported in this paper are: First, the measurement of the increased metabolism; second, the determination of whether or not shivering is necessary for this increase; and third, the relation of skin and body temperature to the onset of shivering.

EXPERIMENTAL

The experimental procedure involving the metabolic measurements on 21 human subjects has been described in the preceding paper. In addition to the respiratory quotient and the heat production, which were computed from the air analysis, the nitrogen elimination was measured in several

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cases. Blood sugar was determined on samples taken just prior to and immediately following the exposure to cold. In some of the early experiments the operator entered the refrigerator immediately after each 10-minute collection period for a time just long enough to determine the pulse rate. This was discontinued later, however, as the importance of the data on this point (since the pulse was always found to be the same or slightly

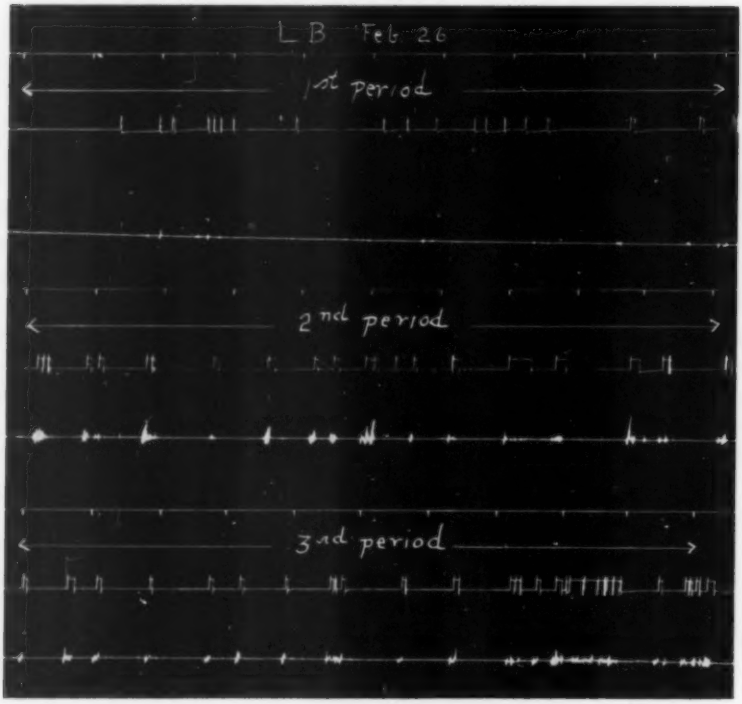

FIG. 1

slower than during the basal) seemed hardly to justify the disturbance caused by entering the refrigerator. Subsequently the pulse was taken immediately after the refrigerator period had ended and while the subject still lay on the cot. Rectal temperatures by clinical thermometer were taken immediately following the basal determination and just after the exposure to cold. The temperature and humidity of the refrigerator were measured by means of a hygrometer.[1]

[1] "Hygrodeik," made by Taylor Instrument Co., Rochester.

Since shivering was one of the important factors in this study, a special effort was made to record it. The six legs of the cot rested on automobile valve springs and a pneumograph was connected to the side of the cot and to the refrigerator wall. The entire pneumograph system was kept under pressure ($\frac{1}{4}$ inch water) to insure sensitivity.

In the earlier trials before the pneumograph was used, a record of shivering was obtained by use of a signal magnet. The subject held a small con-

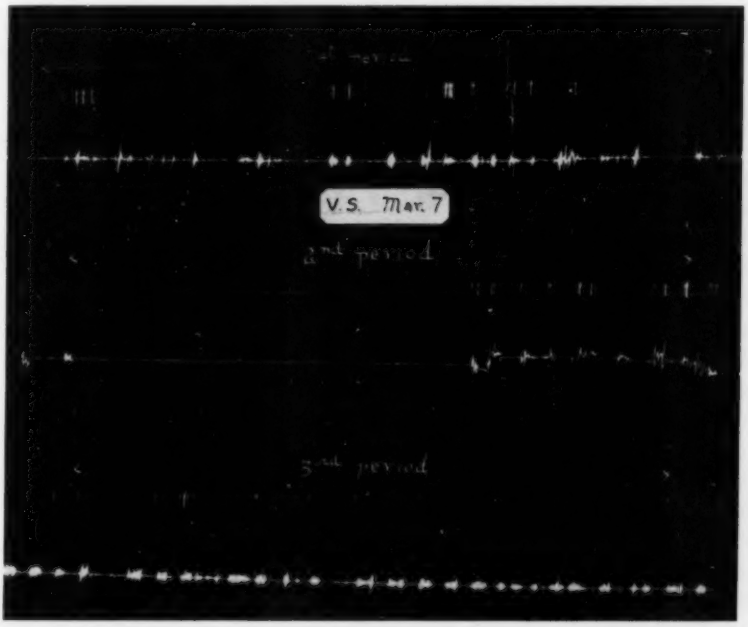

FIG. 2

tact switch which was pressed and held in contact during each transitory period of shivering. To confirm the pneumograph record and to distinguish between shivering and any voluntary movement, the use of the signal magnet was continued throughout. The record of the pneumograph is written directly under the one made at the same time by the signal magnet (Figs. 1 and 2). The top line in all cases indicates the time in minutes. When the switch was held in contact the signal magnet needle was in the "up" position as seen in the photographs. The excellent parallelism between the two records is apparent in the records of shivering.

A sensation of "tightening up" or of increased muscular tonus before actual shivering began was described by the subjects. Some found it difficult to decide whether the chills which "ran up the back" constituted shivering, so that in many of the refrigerator periods there is, therefore, a record of initial shivering as indicated by the signal magnet which is not substantiated by the pneumograph record. The duration of the refrigerator periods was one hour and fifteen minutes.

The onset of shivering under the conditions of the experiment was so gradual as to make impossible any definite record of its actual beginning. The relative impossibility of lying in a relaxed manner in a cold environment has to be experienced in order to be appreciated. The increased "tension all over" experienced by the subjects has no quantitative measurement in this work but cannot be ignored as a source of increased metabolism. Furthermore, very slight tremors in localized parts of the body, too slight to be recorded by the pneumograph, preceded general shivering. After a certain time of exposure it is impossible to avoid vigorous shivering in the leg muscles when the knees are drawn up, even at a time when the subject, lying flat, might well record "no shivering." Shivering often began by very slight and rapid twitches of various muscles in the back and shoulders. There is, therefore, no sharp line of demarcation as to exactly when shivering begins. The signal magnet record is of value in indicating its onset as judged by the subject. Certainly the first slight but definite twitching of the muscles would not be visible to an observer. A translation of a description of the onset of shivering given by Fredericq (11) many years ago is pertinent:

> When the body is exposed to cold, one feels a certain degree of rigidity in all the muscles of the body, especially in the arms and legs. I have thoroughly verified this rigidity in experiments in which I stayed in the cold without clothing; I have been able to ascertain that it is intimately related to the involuntary trembling, which follows reflexly, when the action of the cold is extended further. The tension increases and ends by transformation into intermittent trembling.

The criterion of effective exposure or "the tendency to shiver" which Barcroft and Marshall (12) judged by a tendency of the knees of the sitting subject to come together would indicate that there is an increase of muscular tonus (and of oxygen absorption) which precedes visible shivering. Although the tendency to shiver in their experiment was not noticeable to an observer, the increased tonus may have been responsible for the increased metabolism. This factor is no doubt concerned in those cases of Loewy in which the metabolism increased without noticeable shivering.

No ill effects of any sort were experienced by any of the subjects following the exposure to cold.

Heat Production

The heat production was calculated by use of the Zuntz and Schumberg tables for the calorific value of oxygen, without allowance for the protein metabolism. The error involved is small indeed and, as pointed out by Boothby and Sandiford (13), the method is probably just as accurate for short time experiments on account of the lag in the nitrogen elimination. The calorific value of oxygen per liter was used according to the respiratory quotient obtained. This procedure undoubtedly gives results somewhat too high in those cases in which the superficial high respiratory quotient has involved the use of a higher calorific value. However, it has seemed the only consistent procedure possible. The heat production is given in Table I and in the charts and only a few comments are necessary.

Comparison of 33 basal metabolic rates of adults as obtained in this work with three standards shows that the majority of the results are lower than the prediction value.

	Average deviation in per cent		
	Du Bois formula	Harris-Benedict formula	Dreyer formula
Regardless of sign	8.4	8.6	6.9
Plus	6.2	10.0	10.3
Minus	8.9	8.2	5.8

According to the Du Bois and the Harris-Benedict standards, 26 cases were below the prediction value. The Dreyer formula showed 25 were below. The two trials with subject A.D. have been omitted in the averages as this subject was a case of moderate hyperthyroidism. The basal metabolism of this subject was high to the extent of 11.9 per cent, 13.6 per cent, and 14.6 per cent, according to the prediction value of Du Bois, Harris-Benedict, and Dreyer respectively. Subject A.B.M., though presumably a normal subject, had a high basal rate which in three separate tests averaged considerably above the standards. This average deviation was 9.7 per cent, 14.0 per cent, 15.4 per cent, according to the formulas of Du Bois, Harris-Benedict, and Dreyer respectively.

It is perhaps not surprising that most of the values were found to be lower than the standards. Krogh (14), MacLeod and Rose (15), Hafkesbring and Borgstrom (16), and Du Bois (17) have reported findings which indicate that the standards are too high when the modern methods of rest and quiet are observed.

The parallelism between the heat production and the volume of air breathed is so evident in the charts as to need no particular comment. Though the heat production can be computed quite accurately from the

TABLE I

HEAT PRODUCTION, NITROGEN ELIMINATION, AND BLOOD SUGAR OF SUBJECTS EXPOSED TO COLD

Subject	Heat production per square meter per hr.				Nitrogen elimination per hr.		Blood Sugar	
	Basal	Period 1	Period 2	Period 3	Basal	During exposure to cold	Basal	After exposure to cold
	Cals.	Cals.	Cals.	Cals.	Grams	Grams	Mg. per 100 cc.	Mg. per 100 cc.
A.A.	38.74	58.11	82.67	96.37				
A.A.	40.08	72.17	96.95	82.09				
E.G.*	34.41	36.27	47.07	62.09	.70	.55		
A.B.M.§	39.70	48.69	55.27	66.73	.54	.50	91	97
M.M.*	32.69	40.49	52.91	63.75	.34	.35		
R.E.S.*	35.41	42.14	58.60	69.43			67	65
S.P.	41.19	46.10	47.73	57.59			75	75
W.D.*	40.22	43.27	44.59	69.46			70	71
A.P.*	36.50	46.79	56.67	73.15			85**	76**
J.M.	33.00	42.62	52.68	58.14	.53	.52		
J.R.*	45.55	52.75	58.94	80.37	.48	.65		
A.D.*	41.28	60.15	67.44	82.15	.51	.68	75	77
E.H.*	34.01	62.20	60.07	57.37				
L.B.	37.32	40.37	55.94	61.30				
V.S.	33.34	62.06	47.13	68.17				
T.S.	34.88	37.06	51.93	76.38			80	71
C.H.	38.69	38.71	36.75	38.00			86	72
R.D.*	37.93	38.56	37.85	38.46			81**	80**
E.L.G.*	36.58	37.01	36.68	38.15	.51	.74		
L.M.*	36.33	38.67	38.37	44.34			63	60
H.H.*	35.47	35.34	38.50	47.69			80	77
E.B.	34.94	44.05	43.18	60.40				

* Average heat production of two trials.

** Average of two trials.

§ Average heat production of three trials.

volume of expired air in many cases, such a procedure is not very trustworthy, especially in cases of hyperventilation.

As a general rule shivering increased steadily in vigor and in frequency as the period of exposure continued. Lack of space prohibits the presentation of all the records of shivering which show very clearly the dependence of increased metabolism upon the duration and vigor of shivering.

In order to obtain a roughly quantitative value of the increase in heat production caused by shivering, some actual measurements of these intervals of time, as indicated by the signal magnet, were taken. The periods used are indicated in the following table. The record made by the signal

TABLE II
RELATION OF TIME SPENT IN SHIVERING TO THE INCREASE IN METABOLISM

Subject	Date	Heat Production, increase over basal Per cent	Time spent in shivering* Per cent	Increase in heat production caused by shivering 1% of the time Per cent
A.P.	Feb. 12	68.5	20.8	3.3
" "	Mar. 14	130.8	48.8	2.7
H.H.	Mar. 3	6.2	5.9	1.1**
" "	Mar. 12	63.4	17.1	3.7
L.B.	Feb. 26	64.3	18.5	3.5
R.E.S.	Feb. 20	109.4	18.4	5.9
" " "	Mar. 20	83.4	14.3	5.8
A.D.	Feb. 15	116.5	15.0	7.8
V.S.	Mar. 7	104.5	43.7	2.4
Average				4.0

* Third refrigerator period.
** Shivering not so marked in this test as in the one of March 12.

magnet in these periods is not complicated by any compromise of judgment by the subjects as to whether shivering occurred, for shivering had begun considerably earlier in every case and the record of the signal magnet is perfectly confirmed by the pneumograph record. However, since the accuracy of the signal magnet record depends on the attention of the subject, the pneumograph record is given the more consideration in computing these intervals of time.

In so far as this method of getting at the quantitative effect of shivering on metabolism tacitly assumes the metabolic rate between successive intervals of shivering to be at the basal level, it is of course inaccurate. The variability in the extent of shivering has been brought out early in the dis-

cussion and it is to be understood, of course, that no strict quantitative measure of shivering in these experiments is possible because intermittent shivering, though vigorous, definite, and well-defined in duplicate experiments, is not necessarily identical in its extent or effects. Du Bois (17) imitated as best he could the shivering of a malarial chill and found about the same increase in metabolism (170 per cent) as was observed in that of the patient.

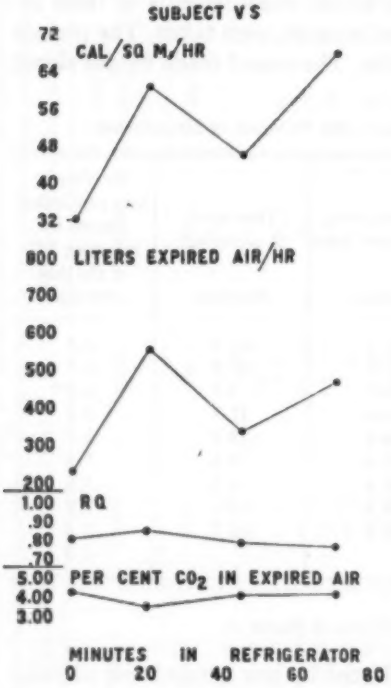

CHART 1

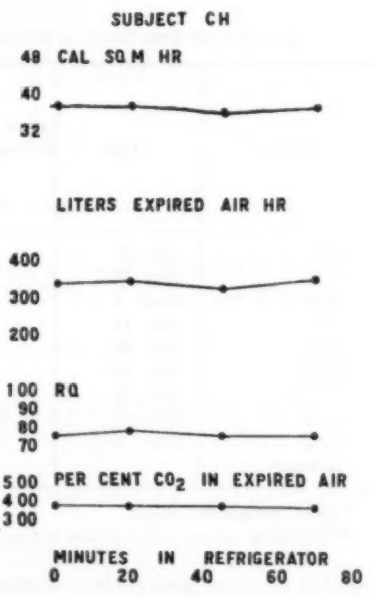

CHART 2

The exceptional behavior of subject V.S. (Chart 1), showing a drop in heat production in the second refrigerator period, is associated with the fact that shivering, which had been very pronounced during the previous half hour, entirely stopped for 6 consecutive minutes, which time constituted 60 per cent of the collection period of the air sample. The subject reported that he did not "feel cold" during this particular interval. The results obtained with this subject are exceptional, as regards the lull in shivering, but quite characteristic as regards the relation between shivering and heat production.

Subject A.A. reached the same peak of heat production in both tests but sooner in the second than in the first. The greater drop in body temperature during the second trial would account for the difference obtained in the final periods of these two days, since an actual cooling off of the body would not be measured by indirect calorimetry. In fact Barr and Du Bois (18) show that a body temperature change during a calorimeter test may be measured by the discrepancy between direct and indirect calorimetry. In so far as a slight drop in body temperature occurred in practically all cases, the above observation applies to all the measurements of heat production. Subject A.A. was in unusually good physical condition which may be related to the great height of heat production attained. Benedict and Smith (19) have shown that athletes have a higher metabolism than non-athletes. Du Bois (17) likewise attaches importance to differences in athletic training. Lusk (20) found the most pronounced reaction to a cold bath with a subject who was a well-trained athlete. Kaup and Grosse (21) report several definite effects as a result of two months' training. These changes were a decrease in body weight and chest circumference along with an increase in chest expansion and vital capacity. Training lowered the blood pressure and increased the circulatory minute volume because of the increased systolic discharge of the heart. The rise of alveolar CO_2 tension was less marked in training. The respiratory quotient both at rest and during exercise was increased by training. The trained subject showed a much quicker return to normal after exercise in respect to heart rate, blood pressure, and oxygen consumption. The efficiency with which exercise was performed was increased.

The temperature and humidity of the refrigerator were not appreciably different in duplicate tests. The heat eliminated by the subject warmed the refrigerator somewhat and, since relatively little water is eliminated through the skin under the experimental conditions, the humidity usually fell.

Variation in duplicate tests is apparently the result of some physiological difference too slight to be detected by comparing duplicate basal rates but which is manifested on exposure to cold. The subjects who showed little or no change in heat production will be considered when subcutaneous fat is discussed.

Respiratory and Pulse Rates

A complete record of respiratory and pulse rates shows in the majority of cases that both decreased slightly during the exposure to cold. This decrease, it is of interest to note, took place while the metabolism was rapidly

increasing. The work of Barcroft and Marshall and of Loewy, above referred to, showed likewise a decrease of the pulse rate and respiratory rate respectively. The failure of the pulse rate to increase does not exclude the possibility of increased secretion of epinephrin since the nervous system of the subject was of course intact. In no case, except J.M., January 19, did hyperventilation involve a faster respiratory rate. Harris and Benedict (22) report a poor correlation between pulse rate and total (basal) metabolism. Murlin and Greer (23) found that a cold bath increased the pulse volume of the dog. Marshall (24) produced marked shivering in a dog by means of a cold bath and obtained only a slight increase in heart rate but a marked increase in output per beat.

Subcutaneous Fat

That subcutaneous fat may be of importance as a factor in heat regulation, seems self-evident. Benedict *et al.* (25) found bigger increases in pulse rate with fat subjects exposed to hot air than with normals. Lusk (26) considers subcutaneous fat as a blanket which delays the chemical regulation of body temperature.

Measurements of subcutaneous fat were taken as prescribed by Franzen (27). These directions call for two measurements on the biceps, four on the triceps, and four on the calf of the leg. Since these measurements were closely alike for a given locality of the body, only one value for each is recorded. In addition to these measurements, three additional ones were taken at points on a transverse line three centimeters above the navel, one directly above, one at the same level at the side, and one 5 centimeters from the middle of the back. The calipers were set at 40 in obtaining the readings as was the case in the triceps and calf measurements. The results are given in Table III. The variation in the distribution of fat and the somewhat questionable significance of the procedure make the actual figures obtained of limited value. The measurements on the body proper and those on the arm and leg are given equal consideration. Though the measurements of subcutaneous fat do not confirm in a quantitative way the reaction of the subjects to cold, it is apparent that the chief reason for the tremendous variation in response to cold of different subjects is the amount of protection afforded by the insulating fat. The differences in metabolism sometimes noted with a subject in duplicate tests can not of course be considered a function of fatness. The simple comparison of weight (kg.) per unit of height (cm.), which gives a general idea of the type of stature of the individual, shows that the particular individuals who give a value of more than 0.45 are also the individuals who show little or no in-

TABLE III
MEASUREMENTS OF SUBCUTANEOUS FAT

Subject	Biceps	Triceps	Calf	Abdomen	Side	Back	Sum
A.A.	15	22	29	19	12	19	116
E.G.	15	25	20	22	26	29	137
A.B.M.	14	21	24	22	11	21	113
M.M.	14	24	23	20	23	21	125
R.E.S.	11	14	21	15	16	18	95
S.P.	10	14	20	15	14	16	89
W.D.	12	19	18	24	20	22	115
A.P.	11	12	17	19	16	19	94
J.R.	14	22	26	20	20	21	123
A.D.	14	23	24	21	21	22	125
E.H.	16	25	17	25	24	30	137
L.B.	11	18	16	18	20	23	106
V.S.	12	18	17	22	22	21	112
T.S.	11	16	18	15	16	21	97
C.H.	15	22	30	34	28	30	159
R.D.	15	27	16	31	33	35	157
E.L.G.	14	29	23	22	23	28	139
L.M.	11	20	23	20	23	29	126
H.H.	13	27	27	32	24	29	152
E.B.	21	20	20	18	19	25	123

crease in metabolism when exposed to cold under the experimental conditions. The only exception to this is subject J.M. Although this subject had a rather large weight per unit of height, the metabolism was markedly influenced by the cold. According to Munk (28) shivering occurs in older persons under conditions which would not cause shivering in younger ones. This being true, it would seem to explain the results obtained on this subject. One very fat subject (Chart 2) showed absolutely no increase in metabolism during exposure to cold. Ebbecke (29), from experiments on human subjects, concluded that the sensation of cold or warmth on the skin depends upon the difference in temperature in the layers of the skin immediately adjacent to the cold and warm receptors and that the temperature sense nerve endings for heat are situated in the deeper layers of the skin, while the cold sense nerve endings lie at the junction of the epidermis and the cutis vera. Inasmuch as subcutaneous fat seems to protect the cold sense nerve endings, the effective fat must lie between them and the surface.

Nitrogen Elimination

The higher nitrogen values obtained (Table I) in some of the cases might seem to indicate an increased protein katabolism during exposure to cold. However, considering the varying conditions, the periods of collection

were rather short to allow close interpretation of the results. The periods of collection varied from one hour and a quarter to two hours and a quarter. An exposure to cold has a marked diuretic effect by decreasing the loss of water through the skin, and by action through the nervous system. The diuresis would tend to give higher nitrogen values by reason of its "washing out" effect. Also, the total metabolism of subject E.L.G., whose nitrogen elimination apparently increased 45 per cent, rose only to 5.9 per cent above the basal during the last refrigerator period. Some of the other subjects whose metabolism increased very greatly show no change in nitrogen elimination.

The experiment was not planned for a special study of the protein katabolism, the above observations being included in a supplementary manner. Also, inasmuch as there was no rigid control of water intake, the results must be only broadly interpreted. The results of Voit (1), cited above, should be considered more significant than these since he collected 6-hour samples for the determination of nitrogen. Chaikoff and MacLeod (30) found no change in nitrogen elimination as measured by 24-hour samples from dogs exposed to cold. Cohn and Gessler (31) have reported similar findings. The results given in Table I are interpreted as indicating no significant change in protein metabolism during exposure to cold.

Blood Sugar

The method of preparing the blood filtrate was that of Haden (32) and sugar determination was carried out as outlined by Benedict (33). No comment on the results shown in Table I is necessary except that the change in blood sugar was in general hardly outside the limit of experimental error. Freund and Marchand (34) found increases in blood sugar in the dog when exposed to cold. Kramer and Coffin (35) found that the exposure of a dog to a low environmental temperature raised the blood sugar only if the exposure continued for a day or more. Shivering did not raise the level of blood sugar. Britton (36) administered insulin to normal cats, and when the blood sugar had fallen to the convulsive level, placed them in cold water with the result that the blood sugar rose and shivering occurred. In cats without active adrenals the blood sugar changes were small and shivering never occurred. They showed no tendency to regain their previous body temperature. The injection of epinephrin raised the blood sugar and restored shivering. This shows a close relationship between epinephrin, blood sugar, shivering, and body temperature control.

Dworkin and Finney (37) produced artificial hibernation in the woodchuck by the administration of insulin. The animal, in profound hypo-

glycemia, lost its power of temperature control and in an environment even moderately cool, passed into a state of hibernation. Injected glucose terminated the condition, produced shivering, and the recovery of body temperature.

However, the nervous control of body temperature can not be dependent upon the blood sugar level, for heat regulation is intact in the phloridzinized dog in spite of the low level of the blood sugar.

The Rôle of Epinephrin in Temperature Regulation

That epinephrin secretion can be increased by cold, and if so increased may affect heat production, seems indicated by the reports of Boothby and Sandiford (38), Hunt and Bright (39), and by Cori and Cori (40). The latter showed that epinephrin did not raise the respiratory quotient of fasting rats although the heat production increased 17.3 per cent. During glucose absorption they found the heat production of rats injected with epinephrin to be increased 16.4 per cent and that the extra heat, in the presence of a carbohydrate plethora, was furnished solely by the oxidation of fat. In experiments on human subjects (41) they found that injection of epinephrin did not raise the arterio-venous blood sugar difference though the blood sugar level invariably rose.

That epinephrin increases metabolism is well established. Evidence that it is secreted in increased amount as a result of exposure to cold is indicated by the work of Hartman *et al.* (42). Hartman and Hartman (43) also found cold to be effective, independent of excitement or muscular activity.

Cannon *et al.* (5) observed a considerable increase in the denervated heart rate of the cat, when exposed to cold, which did not take place when one adrenal was removed and the other denervated.

Demonstration as to the increased secretion of epinephrin was impossible in the experiments of this paper. It seems significant, however, that Cori and Buchwald (44) found from the intravenous injection of epinephrin into normal men that the minimum dosage of epinephrin sufficient to increase the oxygen consumption was twice as great as that necessary to produce hyperglycemia. The unchanging level of the blood sugar during exposure to cold is, therefore, strong evidence that increased secretion of epinephrin did not accompany the increases in metabolism.

The Rôle of Shivering in Body Temperature Regulation

Since shivering is a characteristic response to cold, it may be properly considered as part of the heat-regulating mechanism.

Richet (45) distinguished between a shivering reflex caused by application of cold to the skin and that caused by a cooling of the whole body. The first does not take place with a dog under anaesthesia. The same author (46) showed that reflex shivering took place before there was any appreciable drop in body temperature. In the case of an animal under anaesthesia, however, shivering may not occur until there is a drop in body temperature of as much as 6° C. Sjöström (8) believed shivering to be a reflex initiated by the cold receptors and that skin temperature was the decisive factor for its onset.

Sherrington (47), with dogs, has shown the clearness with which spinal transection separates the body in regard to its response to cold. Dogs with spinal transection placed in ice water showed no sign of shivering behind the transection though there was acute shivering in the region forward from it. Apparently shivering can not be produced as a spinal reflex.

According to O'Conner (48), shivering in cats and rabbits depends upon the temperature of the brain rather than on that of the skin. The animals responded to brain temperature controlled by warming or cooling the carotid vessels. These animals were under anaesthesia, however, and are therefore not comparable to normal animals which shiver when no appreciable drop in body temperature takes place.

It has been shown by Finney *et al.* (49) that the shivering reflex disappears in dogs given amytal when the temperature drop is from 9 to 17° C. depending on the depth of anaesthesia and the condition of the animal. Dworkin (50) recently states that the exact significance of shivering is still not settled, "while very little indeed is known concerning its mode of origin and its nervous control."

The work of Voit indicates a chemical control of body temperature independent of shivering. This work, confirmed by Rubner and Lewaschen, constitutes the old work upon which the conception is based. The experiments were of several hours' duration in all cases and the results are without doubt perfectly trustworthy. Although this work was done before the importance of "extra CO_2" elimination was fully appreciated, the experiments were fortunately of several hours' duration, which minimizes any error of this nature. It should be borne in mind, however, that the noteworthy increases in metabolism occurred at temperatures not far removed from those which produced visible shivering.

The excellent work of Cannon certainly indicates the production of epinephrin in cats when exposed to cold, or when a "heat debt" is incurred by ingestion of cold water. His experiments on men showed that a "heat debt" incurred in the same way, resulted in an increased metabolism

though no shivering occurred. He points out that the room temperature was an important factor in its effect on the extent of the increases obtained. Shivering invariably caused a marked rise in metabolism.

Shivering occurred in cats in which the adrenals were lacking much sooner and for longer intervals than in normal animals. "Thus when the heat-producing service of the adrenal medulla is lacking, the shivering mechanism is resorted to."

The inactivation of the adrenal glands, however, not only removes their "heat-producing service," but also any and all other effects which they may normally exert. Aside from any power to increase the metabolic rate, the effects of epinephrin are characterized by increases in blood sugar, constriction of peripheral blood vessels, and by increases in heart rate and blood pressure. The extent to which these known effects influence metabolic rate is not precisely known. It may also be questioned whether a "heat debt" incurred by the ingestion of cold water involves the same mechanisms as does the stimulus of cold on the skin.

Shivering induced by a cold douche or by exposure of the naked body to cold air takes place independent of any appreciable drop in body temperature. The "heat debts" in Cannon's work apparently caused a definite drop in body temperature as judged by the axilla temperature reported and certainly lowered the temperature of some of the inner organs. "Heat debt" brings about conditions which do not duplicate those caused by exposure to cold air. Shivering, when produced under the two sets of conditions, is brought about in quite dissimilar manners,—one from within and one from without. It is at least conceivable that the factors involved in maintaining a constant body temperature may be used, under the two conditions, to significantly different extents.

The experiments of Johansson and those of Sjöström also involved the determination of CO_2 as a measure of the metabolism. Cannon criticizes their conclusions,—that increases in metabolism without shivering never occur—, in view of the fact that increases in CO_2 output were observed, from 7 to 28 per cent, which were not accompanied by shivering. Evidently these authors as well as Loewy fully appreciated that there was a gradual increase in muscle tension which precedes shivering and which merges into it. They were willing to ascribe to it such increases in metabolism as they obtained not due to visible shivering. The onset of shivering caused by exposure to cold air has been described in detail earlier in this paper and a parallel description taken from the words of Fredericq.

All the periods in which an increased metabolism over the basal occurred without shivering have been listed in Table IV, the average increase being

11.3 per cent. Experiments in which no shivering occurred subsequently have been omitted as there is no exact way of determining how soon shivering would have taken place. Inclusion of such values would not raise the average. Differences between refrigerator periods and the basal metabolism in such cases may represent merely physiological variation and can not with certainty be ascribed to the environmental temperature. It is important to note, however, in this connection, that subject R.D.,

TABLE IV
INCREASES IN HEAT PRODUCTION NOT ACCOMPANIED BY SHIVERING

Subject	Date	Basal Cal.	In Refrigerator	
			Cal.	Per cent increase
R.E.S.	Feb. 20	34.46	41.21	19.6
" "	Mar. 20	36.36	43.06	18.4
S.P.	Feb. 23	41.19	46.10	11.9
" "	" "	"	47.73	15.9
W.D.	Feb. 27	39.26	42.21	7.5
" "	" "	"	42.29	7.7
" "	Mar. 13	41.18	44.32	7.6
" "	" "	"	46.88	13.8
A.P.	Feb. 12	35.67	38.82	8.8
J.M.	Jan. 19	33.59	36.52	8.7
J.R.	Jan. 7	45.43	55.57	22.3
" "	" 31	45.67	49.92	9.3
" "	" "	"	53.97	18.2
L.M.	Feb. 22	35.86	38.48	7.3
" "	Mar. 1	36.80	38.85	5.6
" "	" "	"	38.70	5.2
H.H.	Mar. 12	35.03	36.24	5.2
Average				11.3

February 11, who showed an increase of 9.0 per cent during the last refrigerator period, began to shiver just after the last air sample had been taken.

In order to determine whether increase in muscle tension could account for such increases in metabolism as are shown in Table IV, the following test was made on J.R. who had previously been a subject in many refrigerator trials. This particular experiment was done at room temperature. The basal metabolism was determined in the usual manner. Immediately afterward the subject was instructed to imitate as faithfully as possible the increase in tension which he had experienced during refrigerator periods. This increased tension has been described in some de-

tail above and in this experiment no muscular movement whatever took place. The results are shown in tabular form:

	Basal	Voluntary muscular tension	Per cent increase
Liters CO_2 per hr.	14.17	25.03	76.6
" O_2 per hr.	17.03	23.15	35.9
R.Q.	.832	1.081	
Liters expired air per hr.	338.6	766.3	126.3
Liters expired air per hour per L. O_2	19.9	33.1	
% CO_2 in expired air	4.224	3.306	

Muscular tension alone, without regard to shivering or exposure to cold, may thus increase the metabolism 36 per cent. Making generous allowance for exaggeration in an honest attempt to reproduce the involuntary tension in refrigerator experiments, there is still enough margin left to account for any increases encountered which did not involve shivering. If judged by the CO_2 production, a much larger increase is noted. The great discrepancy between the increase in the CO_2 production and in the oxygen absorption shows the possibility of drawing erroneous conclusions from CO_2 measurements over short periods. Incidentally, the data in this test exhibit all the earmarks of *Auspumpung* described in the first paper of this series.

The important point, however, to the general subject of this paper is that by a failure to relax, the oxygen consumption may be increased 36 per cent. The relative impossibility of relaxation when exposed to cold has already been mentioned. It seems, therefore, logical to attribute the increase in metabolism in those periods listed in Table IV to increased muscular tension.

The term "no shivering" is not usable as a critical measure of condition. Whereas "shivering" designates a more or less definite condition invariably associated with increased metabolism, "no shivering" may indicate anything from complete relaxation, with no increase in metabolism, to a state of muscular tension involving a very pronounced increase in metabolism. This is a point to be stressed since the chief objection to the claims of Loewy, Johansson, and Sjöström is that definite increases in metabolism occurred with "no shivering."

Shivering may well be considered to be substituted for mere muscle tension increase when the latter no longer suffices. This is essentially the idea put forth by Fredericq but which has been ignored by those who have, tacitly at least, used "no shivering" to mean no increase in muscle tension.

In consideration of the gradual and progressive changes in muscle tension which characterize the period preceding visible shivering, the burden of proof as to the existence of other factors would seem to lie with those who are not willing to ascribe to these changes the metabolic increases noted. Cannon points out that Loewy had to assume an increase in muscular tension in many cases where metabolism increased without shivering. A much more improbable assumption would be that increased muscular tension did not exist.

The belief that increased muscular tension is responsible for the increased metabolism which occurs without shivering, does not, of course, exclude the idea of epinephrin production. Cramer (51) believes epinephrin to be important in heat regulation but that its effect is brought about entirely through the sympathetic nervous system. The constancy of the blood sugar, however, argues against epinephrin production.

The Stimulus for the Onset of Shivering

An effort was made to include in this work a small contribution regarding the stimulus for the onset of shivering. Since the rectal temperatures, taken before and after exposure to cold, had previously shown almost invariably a definite decrease, a few experiments were performed during which a record of the rectal temperature was obtained by means of a resistance thermometer and recording galvanometer. These temperatures, accurate to .01° C., were taken every two minutes. The thermometer was inserted a distance of 12 centimeters, this distance being the same in all cases and determined by a bulb of hard rubber fastened to the thermometer. In one experiment the subject lay on a cot in the refrigerator while the rectal temperatures were being recorded. A record of shivering was obtained in the usual manner. In a second experiment, the same subject lay on a cot at room temperature (24° C.) during which time the rectal temperature was recorded as above. The data obtained with subject J.R. are set forth in Chart 3. Closely similar results were obtained with another subject.

Two points of importance are to be noted. First, the drop in body temperature, up to the time shivering begins, is the same as obtains when the subject lies down in a warm room. A drop in body temperature occurring on lying down at room temperature has been reported by Benedict and Slack (52). Their results also showed that rectal temperature is an excellent indication of the temperatures of all other parts of the body exclusive of the skin. Second, the slow and gradual drop in body temperature is not lessened by vigorous shivering. From these results it seems certain

that even a slight drop in body temperature can not represent the stimulus for shivering. In both the above cases the shivering was very marked during the last 10 minutes and was taking place when the body temperature had not dropped any more than in the parallel experiment done at room temperature. The above observations illustrate the striking degree of perfection of the heat regulating mechanism. This subject had been in the refrigerator several times before when the metabolism had been determined and it is safe to assume that during the last few minutes of this particular test the heat output was nearly doubled. That the body temperature was not raised the least bit by marked shivering extending over a period of from 15 to 30 minutes, is surprising.

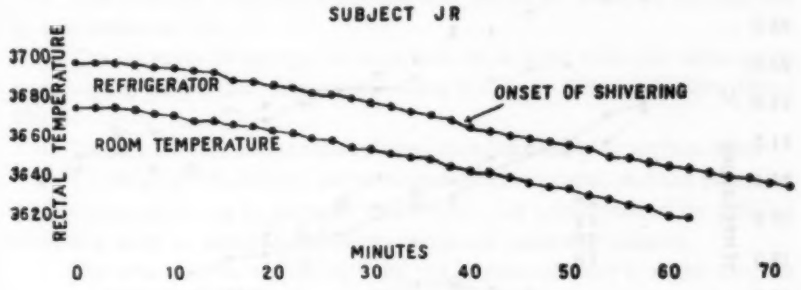

CHART 3

Shivering and Skin Temperature

In an effort to correlate skin temperature change with the onset of shivering, a special thermopile was made to record the surface temperature at various parts of the body. This contact thermopile consisted of four junctions of copper and constantin, and was carefully calibrated.

Benedict and Slack (52) pointed out that surface thermometers are subject to the temperature of the environment as well as to the skin temperature and are "at best unsatisfactory and inaccurate." The unfavorable conditions of use in the work here reported increase still further the uncertainty of the values obtained since the environmental temperature of about 2° C. is very much below that of the body or of the skin. Considerable effort was consequently expended in preliminary trials to find the time that the thermopile should be applied to the skin under the conditions of the experiment. Since the reading on the chart increased only slowly after the thermopile was in contact with the skin for 25 seconds, this was the time of contact used. Readings were taken every two minutes (Chart 4).

The subject sat down in the refrigerator, stripped to the waist, and applied the thermopile according to signals given by a light. As was the case in other experiments, the onset of shivering was not sudden and could not be designated accurately. The definite involuntary movement or twitchings of muscles (usually those about the shoulders) was considered the beginning of shivering. The second test made with subject J.R. followed the completion of the first by an interval of only about 20 minutes, the subject being exposed to a room temperature of 23° C. during this interval. Apparently the skin temperature had not returned to normal, which partly accounts for the lower initial readings in the second test. The thermopile

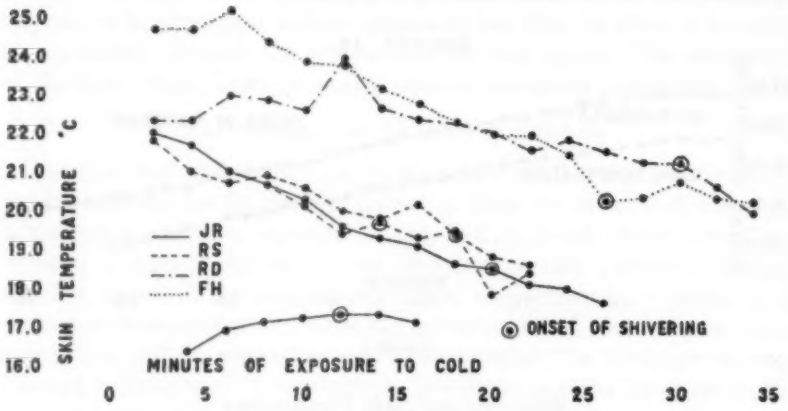

CHART 4

was exposed during this interval to the refrigerator temperature so that the lag of reaching the skin temperature would also tend to make the first readings in the second period low.

The energy metabolism of subjects J.R. and R.D. is shown in Table I. Subject R.D. did not shiver in those experiments as contrasted with subject J.R. and it is significant that the former reached the point of shivering, when stripped to the waist, only after a considerably longer exposure to cold. His skin temperature was apparently somewhat higher when shivering had definitely begun. Emphasis of this small difference, however, is prohibited by the inaccuracies inherent in the method of measurement. It seems reasonable to conclude that when normal persons exposed just previously to room temperature and normally clothed are exposed to cold under the conditions described above, shivering definitely begins when the skin attains a temperature of approximately 19.0° C.

Summary

A study of the data obtained on 21 human subjects in a basal condition, exposed to an environmental temperature of about 2° C. for one hour and a quarter, permits the following conclusions:

1. There is no change in the protein metabolism corresponding to the increase in energy metabolism.
2. The blood sugar level remains unchanged, indicating that increased epinephrin secretion is not involved.
3. There is no parallelism between the energy metabolism and pulse rate or respiratory rate, but distinct parallelism between heat production and respiratory volume.
4. The reaction to cold in a general way varies inversely as the amount of subcutaneous fat.
5. The increase in energy metabolism considered with the time spent in shivering shows that intense shivering increases the metabolism about 400 per cent.
6. The increase in metabolism shows no relationship to surface area.
7. The heat production is proportional to the amount and the intensity of shivering, and any increase in metabolism not accompanied by definite shivering may be justly ascribed to increased muscular tension.
8. The stimulus for shivering does not consist of even a slight drop in body (rectal) temperature.
9. Shivering begins when the skin attains a temperature of approximately 19° C.

Acknowledgment

The writer is pleased to express his thanks to those who so unselfishly volunteered to serve as subjects. Mr. J. W. Karr assisted materially in the careful determination of the blood sugars. It is a pleasure to acknowledge the continual guidance and suggestions of Professor J. R. Murlin.

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SOME EFFECTS OF RESTRICTED PROTEIN INTAKE ON THE ESTROUS CYCLE AND GESTATION IN THE RAT*

By

H. R. GUILBERT AND H. GOSS

(From the Division of Animal Husbandry, College of Agriculture University of California, Davis)

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THE idea that any dietary deficiency may be a limiting factor in reproduction has been developing for a number of years, and experimental evidence, which has been accumulating, supports this conception. Thus Evans and Bishop (3) showed that quantitative undernutrition, induced by limiting the intake of a normal stock diet, seriously interfered with sexual maturity and ovulation in rats.

Deficiencies of single vitamins have been shown, by several workers, to affect reproduction. Inadequate vitamin A impairs the female reproductive system so that fertilization and implantation often fail. In regard to this Evans (6) states,

> A level of inadequate vitamin A can be secured, denoted by continuous cornified cell vaginal smears, during which estrus and ovulation occur and are, in fact, fairly frequent. This is only demonstrable by the continuous presence of the male, for no changes occur in the vaginal cell types. Four-fifths of the copulations eventuate in failed implantations. A great many of them fail to establish the condition of pseudopregnancy since another estrus occurs in 5 days.

Atrophy of the testes and ovaries of pigeons results from vitamin B (B & G) starvation according to McCarrison (11) while Parkes and Drummond (12) found that sterility followed quickly in male rats deprived of vitamin B. In female rats vitamin B deficiency results in cessation of estrus and the ovulatory function has been shown to be more sensitive to this lack than general bodily nutrition (Evans and Bishop, 3). That the vitamin B requirement for lactation is greatly in excess of that necessary for optimum growth has been shown by Evans and Burr (7) and by Sure (13).

The well known relation of the fertility vitamin E to reproduction need not be discussed here except to note that sterility of females resulting from this deficiency is not due to impairment of the ovaries or ovulation but to a characteristic disturbance occurring in gestation, which results in the death and resorption of the fetus (Evans & Bishop, 4). Inadequate vitamin

* The experimental work reported in this paper became coöperative with the United States Department of Agriculture July 1, 1929.

E leads eventually to destruction of the seminiferous epithelium of the male (Evans & Burr, 5).

Evidence is found in the literature that mineral deficiencies may be involved in reproductive disturbances. Naturally occurring deficiency of calcium and phosphorus in the food of domestic livestock has been cited by Tuff (16), who states that experience in badly affected areas of Norway shows unmistakably a relation to reproduction, indicated especially by failure to ovulate. Difficulty in reproduction in cows which was characterized by premature birth of the calves has been reported by Hart and associates (9) and attributed by them to a ration which was deficient in calcium and vitamin A.

Cessation of estrum in cattle under experimental conditions, on rations deficient in phosphorus, was reported by Eckles, Becker, and Palmer (2). Similar observations have been made on cattle grazing phosphorus-deficient veld in South Africa (Theiler, Green and Du Toit, 15), (Du Toit and Bisschop, 1).

Evans and Bishop (3) found that fat-free diets fed to rats interfered with normal ovulation, but no such interference was attributed to carbohydrate-free diets. Qualitative undernutrition with regard to protein resulted in cessation of ovulation.

Our attention has been directed particularly to the effect of phosphorus and protein deficiencies on reproduction, during the course of an investigation on the factors involved in the variable calf crop obtained in range cattle in California. Analyses of range forage collected at different seasons of the year have shown strikingly that most species of forage become deficient in protein and phosphorus during the long drought period, and frequently the ratio of calcium to phosphorus is extremely wide. Since the naturally occurring deficiencies of protein and phosphorus were found simultaneously, an investigation was initiated with rats for the purpose of extending our general knowledge of the independent effects of these deficiencies. The results of experiments on the effects of varying the calcium and phosphorus intake on the estrous cycle and reproduction in the rat have already been reported (Guilbert & Hart, 8).

General Procedure

The seventy-five female rats used in these experiments were selected at weaning time (21 days of age) and divided into groups. They were kept in metallic cages equipped with false screen bottoms containing 3 meshes to the inch. From one to three rats were kept in each cage.

All of the animals were allowed a normal protein intake until sexual

maturity (about 50 days of age) when some groups were changed to the restricted diets, while other groups were not changed to restricted diets until they had attained an age of about 100 days. It was thus possible to study the effect of diet upon the functioning of ovaries which had been allowed to develop normally.

Following sexual maturity, the stage of the estrous cycle was determined by daily microscopic examination of the vaginal smears.

TABLE I

COMPONENTS OF THE DIETS
(grams per 100 grams diet)

	Diet 13	Diet 16	Diet 26	Diet 27	Diet 28	Diet 29	Diet 30	Diet 31
Casein	4.0	—	—	—	15.0	—	—	3.5
Whole milk powder	3.0	3.0	—	—	—	—	—	—
Egg albumin	—	—	10.0	1.0	1.0	1.0	—	1.0
Wheat gluten flour	—	—	10.0	2.0	—	—	—	—
Corn starch	59.2	62.9	55.0	72.0	62.0	76.5	77.5	73.0
Whole wheat	25.0	25.0	—	—	—	—	—	—
Alfalfa leaf meal	—	—	5.0	5.0	—	—	—	—
Butter	5.2	5.2	6.0	6.0	7.0	7.0	7.0	7.0
Cod liver oil	—	—	1.0	1.0	2.0	2.0	2.0	2.0
Wheat germ oil	—	—	1.0	1.0	1.0	1.0	1.0	1.0
Yeast	—	—	—	—	7.0	7.0	7.0	7.0
Yeast extract*	—	—	7.0	7.0	—	—	—	—
$CaCO_3$	1.5	1.5	—	—	—	—	—	—
NaCl	0.8	0.8	—	—	—	—	—	—
KH_2PO_4	1.3	1.6	—	—	—	0.5	0.5	0.4
Salt mixture	—	—	(4) 5.0	(4) 5.0	(6) 5.0	(6) 5.0	(6) 5.0	(6) 5.0
Crude protein, per cent	7.0	3.5	16.5	4.1	17.5	4.9	4.0	8.2

* Northwestern dried yeast was first extracted with cold 70% alcohol, acidulated with acetic acid, then with hot 70% alcohol, and the combined extracts evaporated in vacuo to a volume such that 1 cc. was equivalent to 1 gram of yeast.

Three groups of diets were employed in which the sources of protein varied. The per cent of protein in the restricted diets varied from 3.5 to 8 per cent. The composition of the diets is shown in Table I.

Phosphate salts were added to the diets to compensate for the reduction coincident with the reduction of protein, thus maintaining the phosphorus content practically constant. The fat-soluble vitamins were furnished in abundance by butter, cod liver oil and wheat germ oil, or as in the case of diets 13 and 16, by butter and whole wheat. Seven per cent of yeast or

yeast extract is known to supply adequate amounts of the vitamin B complex for growth and ovarian function on diets otherwise poor in vitamin B. Diets 13 and 16 are probably poorest in regard to the vitamin B complex, but the appetites of the animals and the difference in the supply effected by the addition or removal of 4 per cent casein would not appear to account for the difference in results secured on these diets.

Salt 4	Per cent	*Salt 6*	Per cent
NaCl	4.05	NaCl	5.00
$MgSO_4$	12.60	$MgSO_4$	5.00
Na_2HPO_4	23.00	Calcium lactate	29.93
KH_2PO_4	17.50	$CaCO_3$	26.92
$CaCO_3$	9.30	Na_2HPO_4	10.10
Calcium lactate	30.80	KH_2PO_4	20.00
Ferric citrate	2.70	Ferric citrate	3.00
KI	0.05	KI	0.05
	100.00		100.00

In diets 13 and 16 the protein is derived from milk and wheat, in diets 26 and 27 from egg albumin, wheat and a small amount from alfalfa leaf meal and from the yeast extract, while in diets 28 to 30, the protein is furnished by yeast and egg albumin or yeast, egg albumin and casein.

Experimental

A group of nine rats were fed upon the stock diet[1] from weaning until 55 days of age, at which time each rat had had two or more periods of estrum. They were then fed diet 13 containing about 7 per cent protein for a period of 40 days, after which time the protein intake was decreased to 3.5 per cent by replacing 4 per cent of casein by corn starch (Diet 16). The data from this group are summarized in Figure 1, by means of a composite growth curve and a graphical presentation of the estrous cycle history.

There was a slight break in the growth curve upon changing from the stock diet to diet 13, but considering the level of protein fed, the rate of growth was good. Some of the rats missed 1 to 3 periods of estrum immediately following the change of diet but later practically all were having cycles regularly at 4- to 6-day intervals. Upon changing to diet 16 containing about 3.5 per cent protein there was a loss of weight followed by 40 days of maintenance, then a slight decline. Estrus ceased in 5 of the 9 rats within a short time after the change to this diet and the remainder

[1] The stock diet is the McCollum normal diet consisting of, whole wheat 67.5%, casein 15%, whole milk powder 10%, butter 5.2%, $CaCO_3$ 1.5%, and NaCl 0.8%.

had irregular and long cycles. During the intervals in which there were no manifestations of heat, the vaginal smear exhibited the typical diestrous characteristics.

A group of 11 rats were fed diet 26 (16.5% protein) from weaning time until 105 days of age, at which time they were changed to diet 27 (4% protein). The data from this experiment are shown in Figure 2.

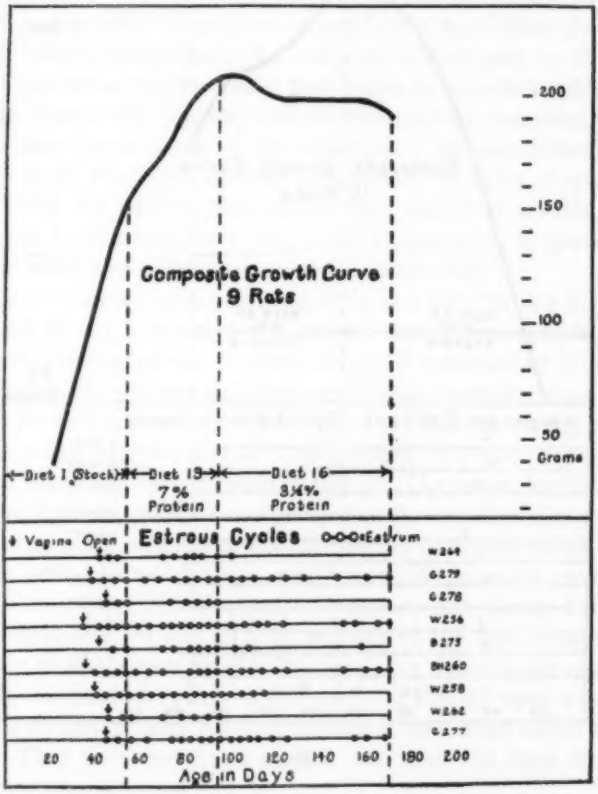


FIG. 1

Diet 26 produced very satisfactory growth, the rats attaining an average weight of 225 grams at 100 days of age. The time of opening of the vagina was somewhat delayed, particularly in 2 of the 11 rats. Upon changing to diet 27 there was an immediate loss of weight, followed by a period of 20 to 30 days in individual rats during which there were slight losses. After

this time, loss in weight was rapid until some of the rats reached a weight only 50 per cent of that attained on diet 26, and were in critical condition. Seven of the 11 rats ceased having estrum within a short period after changing to the low protein diet. The remainder continued for a longer

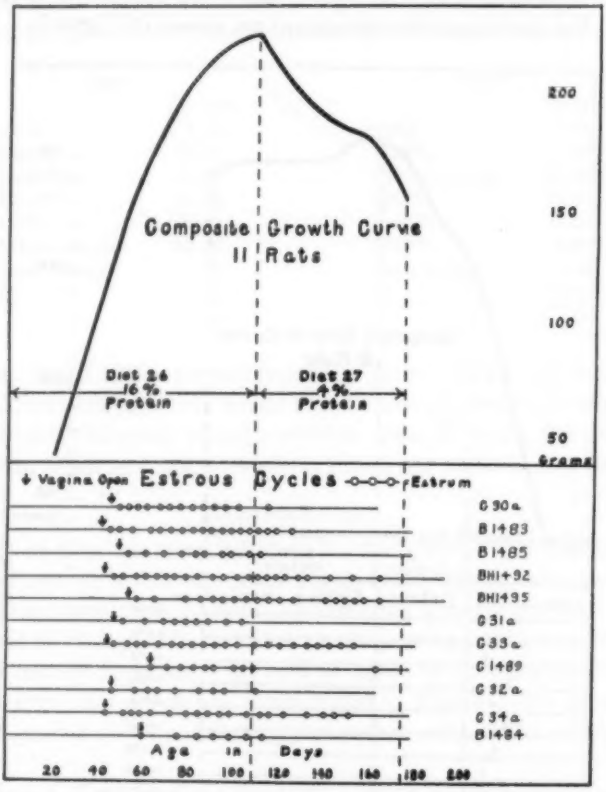


FIG. 2

period as indicated in Figure 2, but all ceased having cycles for a significant length of time before the end of the period.

At the close of the period shown in Figure 2, two rats were autopsied, 4 were changed back to diet 26 and for the remaining 6 rats the protein in the diet was increased by replacing 5 per cent of corn starch with egg albumin. This addition did not lead to improvement of body weight, over a period of 35 days, and the rats were then given diet 26.

There was a rapid recovery in weight on diet 26 in all except one rat which developed pulmonary infection and died. Two individuals did not come into estrum even though they regained or surpassed the weight originally attained on diet 26, and appeared to be in thrifty condition. The total period, prior to autopsy, during which there was no sign of estrus for each of these rats, was 155 days. The remaining rats returned to more or less regular cycles upon regaining the weight lost during the period on the low-protein diet. Several rats began losing hair either shortly before or after being changed from the low-protein diet back to diet 26. New coats of hair developed soon after they began to recover weight on diet 26.

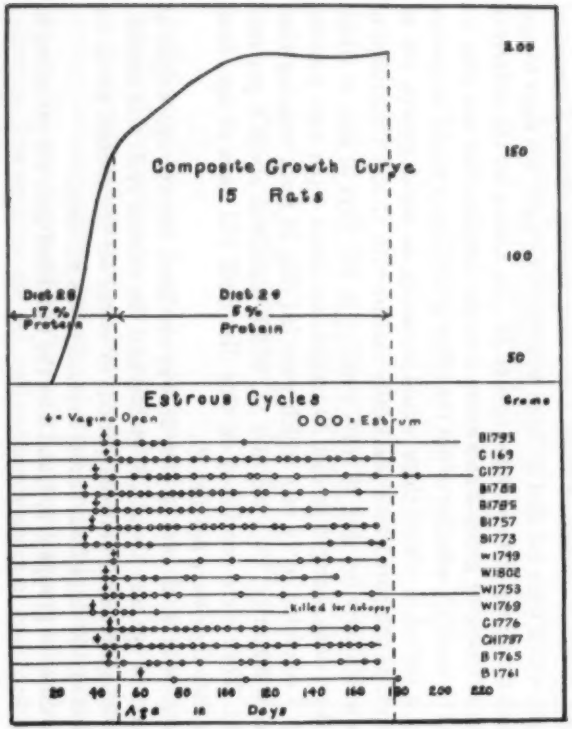
At the close of this recovery test all animals were autopsied. The cessation of the estrous cycles in this experiment was coincident with large losses in body weight, in contrast to a similar cessation of estrus on diet 16 on which the animals maintained their weight at a little under 200 grams and for the most part were fat and apparently in good condition, although there was slightly less protein in their diet.

In the third group of experiments fifty-four rats divided into 4 groups were used. Group 1 consisted of 8 rats fed diet 28 throughout the experiment and constituted the controls. Group 2 consisted of 15 rats which were fed diet 28 from weaning time until sexual maturity when they were fed diet 29 which contained about 5 per cent protein. Group 3 consisted of 8 rats which were fed diet 28 from weaning time until 105 days of age, at which time they were changed to diet 29. The reason for the difference in time of changing group 2 and group 3 to the deficient diet was that we wished to ascertain whether or not the effect of deficiency would be greater during a period in which there would normally be rapid growth (50 to 100 days) as compared with practically mature rats. Group 4 consisted of 9 rats which were fed diet 28 from weaning to 105 days of age, at which time they were changed to diet 30 containing about 4 per cent protein. Group 5 consisted of 14 rats which were fed diet 28 from weaning time to sexual maturity, then fed diet 31, which contained about 8 per cent protein. They were continued on this diet until 152 days of age, from which time they were fed diet 30.

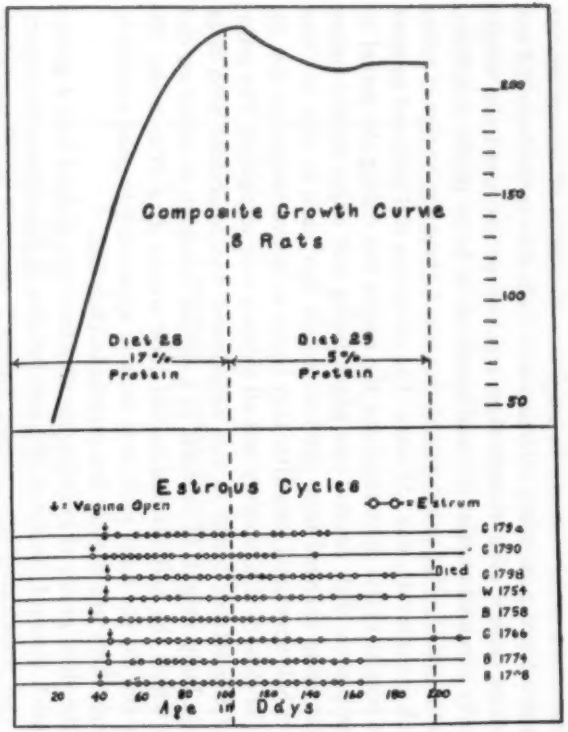
The growth of all rats on diet 28 was excellent, the average weight at 100 days of age was about 230 grams, and the estrous cycle was normal. The average interval between periods of estrus for the control group for a period of 160 days from sexual maturity was 5.1 days.

The data for groups 2, 3, 4, and 5 on the restricted diets are presented in Figure 3.

Upon changing from diet 28 soon after sexual maturity to diet 29, con-

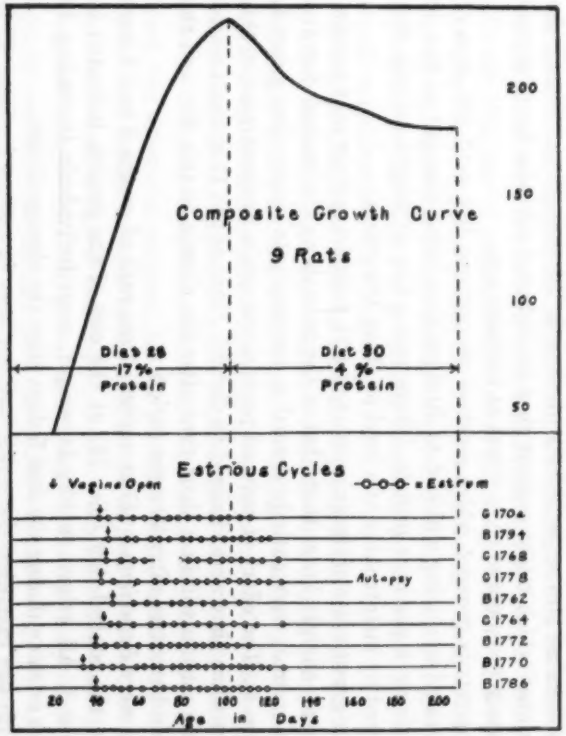


GROUP 2

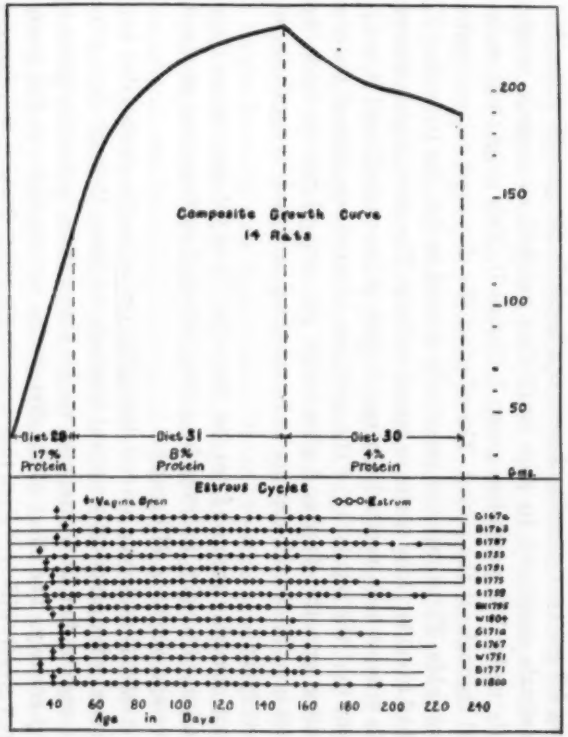


GROUP 3

FIG. 3



GROUP 4



GROUP 5

FIG. 3

taining 4.9 per cent protein, there was a break in the growth curve, but the animals continued to grow until they reached an average weight slightly under 200 grams (see group 2, Figure 3). This weight was maintained throughout the period. The effect of the diet upon estrous cycles was variable. Three rats had practically normal cycles, the remaining 12 rats had irregular, long cycles or ceased entirely. The estrous cycle history of this group was in marked contrast to that of the controls and it is significant that the regularity of the cycle was affected even though the diet permitted growth. Four and nine-tenths per cent of protein from the sources used in diet 29 appears to be marginal in regard to regularity of estrous cycle.

Upon changing group 3 from diet 28 to diet 29 at 105 days of age there was a loss of weight, followed by a long period of maintenance at about an average weight of 210 grams.

The estrous cycle was normal during the period on the control diet and continued with practically normal intervals for from 25 to 60 days after the change to diet 29. Subsequent to this time estrum became irregular or ceased entirely. The effect of the diet on the estrous cycles in this group was not greatly different from that occurring in group 2 which was changed to the low protein diet at sexual maturity. However, it appears that there was somewhat greater persistence of estrus in the younger rats than in the older ones which might suggest that the internal stimulus for ovulation was more pronounced than it was at the later age.

The rats in group 4 which received the control diet until 105 days of age ceased ovulating within 5 to 20 days after being changed to diet 30 containing 4 per cent protein. There was a loss of weight on this diet followed by maintenance at a level of about 180 grams.

The growth rate of the animals on diet 31, containing 8 per cent protein, was only slightly lower than that of the animals on the control diet and these animals were entirely normal in estrous cycle history (see group 5, Figure 3). The effect of reduced protein was again demonstrated in this group when they were changed to diet 30. Eight of the 14 animals ceased having estrum within a short time after the change to this diet and the remainder ceased after a longer period.

Recovery Experiments: Four animals from each of groups 3 and 4 were given the control diet (diet 28) at the end of the periods indicated in Figure 3. All gained rapidly in weight, most individuals increasing 30 grams or more during the first 5 days after the change of diet.

With one exception these rats had not been in estrum for from 65 to 105 days, yet they came into estrum in 4 to 6 days following the change of diet.

Breeding Experiments: Following the periods indicated in Figure 3, the animals in the control group fed diet 28, and those of group 2, fed diet 29, which came in estrum, were tested for fertility.

Of the control group of 8 females, all were bred one or more times. Four of the females failed to mate during estrum from one to three times before a positive mating was obtained. There were a total of 14 positive matings as shown by examination for plug and sperm in the vagina. Twelve of the 14 positive matings resulted in the placental sign (R.B.C.) at the 13th to the 15th day following coitus. Ten of these pregnancies terminated in litters of from 5 to 12 young which were apparently normal and averaged in weight from 5 to over 6 grams each. Of the two additional pregnancies, one litter of 2 dead rats was delivered 4 days later than the normal time of parturition and the other litter consisted of 7 rats, 5 of which were deformed and were born dead. One positive mating was followed five days later by the recurrence of estrum. Pregnancy was later established in this rat. In one female red blood cells appeared in the vaginal smear on the 12th day following coitus and no litter was born. Thus litters were obtained from all but one rat, and for the most part they were normal in appearance and weight at birth.

It was recognized that 7 per cent of yeast as the principal source of vitamin B in the diet would be deficient for lactation, and an additional 1 to 1.5 grams were fed to each female daily, except in 2 cases, beginning with the 20th day of gestation. However, all except 3 litters died within a few days following parturition, the mothers apparently failing to lactate. Diet 28 was excellent for growth and normal rythmic functioning of the ovaries, fair for fertility and gestation, but inadequate for lactation even though supplemented with yeast.

In the breeding tests with the animals in group 2, (fed low protein diet, 29) there were 17 instances of failure to breed when during estrum they were placed with a normal male, positive mating never being obtained in several cases. Fourteen positive matings were secured. In 5 of these positive matings, blood appeared in the vaginal smear at the 8th to 12th day following coitus and no litters were born. That pregnancy had been established was shown on autopsy, by the presence of placental cites in the uterus. One animal had red blood cells in the vaginal smear at the 14th day. There was no sign of littering on the 23rd day following mating and there had been a loss of 20 grams in live weight during the preceding 4 days. It was therefore thought that resorption was occurring. The animal was autopsied and 6 live fetuses weighing 2 to 3 grams each were found in the uterus. This was the only case of young being carried alive full time,

resulting from the 14 positive matings in this group. In the other 8 positive matings, fertilization either was not accomplished or else very early death of the embryos occurred, as there was no sign of red blood cells in the vagina, no litters resulted, and either the diestrous smear persisted or the animals came back into estrum within a few days after mating. One animal was bred and came back into estrum and was rebred 5 days later. Red blood cells appeared in the vaginal smear 8 days from the second mating. Four days later the animal was in estrum and mated. This mating was followed by 2 more recurrences of estrus and matings at 5- and 7-day intervals respectively. Estrus occurred more or less regularly for sometime but no further matings were attempted. The repeated matings and recurrence of estrus in this rat together with similar observations on several others indicated that the condition of pseudopregnancy was not induced.

Eight animals from groups 3 and 4 were bred after the recovery period on diet 28. Positive matings were secured usually after 1 to 3 periods of estrum, and they were then given low protein diet 30, during gestation. Of these 8 rats, 2 had blood in the vaginal smear at the 10th day following mating, no litters were born and the animals did not come back into estrum. Five rats had the placental sign (R.B.C.) at the normal time. Two of these died about the time for parturition, autopsy revealing young which were subnormal in weight, in the uterus of each. One rat gave birth to a litter of dead young, 5 days after normal parturition time. One female gave birth, at term, to subnormal young and another female, given stock diet the last five days of gestation, gave birth to living young which were only slightly subnormal in weight. The mating of the remaining animal apparently did not result in fertilization. A female from group 3, diet 29, which was not included in the recovery experiment, was bred. On the 11th day following copulation there was blood in the vaginal smear and what appeared to be an embryo and membranes were removed from the vagina. The animal was autopsied, and four live embryos were found in the uterus. In a fifth placental cite, in the right horn, nearest to the cervix, no membranes or embryo were found, and it appeared that this was the point of origin of the tissues found in the vagina. Histological sections verified that the material found in the vagina was embryological tissue.

In the cases in which red blood cells appeared at the 8th to 12th day following coitus, the vaginal smear was characterized by a more copious flow of blood than the normal placental sign which appears from the 13th to the 15th day, or that of pseudopregnancy. In some cases it amounted almost to hemorrhage at first, and persisted only a few days, while in other cases the bloody smear occurred continuously up to the 23rd day after

mating. This observation, together with autopsy findings, indicates that the death of different individuals in the litter may not occur at the same time.

Histology of the Ovary: Upon autopsy of animals which had not been in estrum for 60 days or more, large corpora, and also a large amount of yellow or straw-colored material was observed on the surface of the ovaries. Some follicles were also visible. Although we do not know of data on the length of time for the involution of the corpus luteum in the rat in the absence of estrum, we were surprised to find corpora of comparatively large size persisting. It was computed from the data of Long and Evans (10), in regard to the number of corpora in the ovary of normal rats, that complete involution must occur in about 70 days. We, therefore, sectioned several ovaries to ascertain the character of these corpora and also the nature of yellow pigmented substance. The latter was found to be associated with the amount of interstitial tissue. This was found in greatest amount in individuals which had not been in estrum for 150 days, but was also found in ovaries of females which had not been in estrum for as short a time as 30 days.

In the animals autopsied 60 to 70 days after the last evidence of estrum, as many as 20 to 30 corpora were found in a single ovary, the largest of which varied from 600 to 800 microns or from one-half to two-thirds the size of the normal, full-sized corpus luteum. Some of the corpora were still well vascularized and did not have the profuse connective tissue ingrowth, associated with regressing corpora. Others were poorly vascularized and there was abundant connective tissue. A considerable number of follicles of about one-half mature size, numerous smaller follicles, and comparatively few primordial follicles, were found. Many of the follicles were undergoing atresia.

It is not clear from the data at hand whether there was persistence of the corpora from the last heat periods or whether new corpora were forming without ovulation such as Swezy and Evans (14) describe during pregnancy in the rat.

Food Consumption: The question naturally arises as to whether reduced protein did not affect palatability to the extent that the level of food consumption became an important factor in the results secured. Since the food cups employed do not entirely prevent waste, the figures on food consumption include a variable amount of waste, and rats on the deficient diets tended to spill more than the controls. However, when the amount became excessive it was taken into account.

The lowest daily intake per rat was 9.2 grams, and occurred on diet

27 which caused rapid decline in weight. The amount wasted was small, and the daily consumption was only slightly under 5 per cent of body weight. On the other restricted diets the food intake varied from 12.5 to 14.8 grams daily and averaged 6.4 to 7.0 grams daily for each 100 grams live weight. Quantitative undernutrition, therefore, does not appear to have influenced the results in these experiments.

Summary

1. Diets containing 3.5 to 5 per cent protein resulted either in cessation of estrus or in long and irregular cycles.

2. Diets containing 7.5 to 8 per cent protein permitted growth at only slightly less than the normal rate and the estrous cycle was normal.

3. An inadequate level of protein was secured which permitted estrus in some individuals. These animals were subjected to breeding tests but no litters were produced. The lack of fertility was of 3 general types: (a) Repeated failure to mate when placed, during estrum, with normal males. (b) Apparently infertile matings with recurrence of estrus, the condition of pseudopregnancy not being induced. (c) Fertile matings followed by death of the embryos, the incidence of which was denoted by the appearance of blood in the vagina on the 8th to the 12th day following coitus. One definite case of abortion was recorded.

4. There was no marked difference in the effect of restricted protein intake on estrous cycle in rats placed upon the deficient diets at 50 days of age, as compared to practically mature rats.

5. In nearly every case, females which ceased ovulating on deficient diets returned to normal cycles within a short time after being changed back to the control diets.

6. Food consumption data indicate that quantitative undernutrition was not a factor in the results obtained.

7. Histological examination of the ovaries revealed as many as 20 to 30 corpora, many of which were one-half to two-thirds the size of a normal, full-sized corpus luteum, remaining as long as 70 days after the last period of estrum. There were also numerous large follicles and also smaller follicles many of which (both large and small) were undergoing atresia. There were few primordial follicles and an abnormal amount of interstitial tissue. It was not clear from the data available whether or not new corpora were forming or whether there was unusual persistence of the corpora formed during the last periods of estrum.

Acknowledgement: The authors are indebted to Dr. H. H. Cole for the histological examination of the ovaries.

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A METHOD OF DETERMINING THE BIOLOGICAL VALUE OF PROTEIN IN THE STUDY OF AVIAN NUTRITION*

By

J. L. St. John, O. Johnson, J. S. Carver, and S. A. Moore

(From the Divisions of Chemistry and Poultry, Agricultural Experiment Station, Pullman, Washington)

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THE determination of the optimum level of protein which should be used in poultry rations and the determination of the relative value of protein supplements from various sources are problems of practical interest to the poultry industry. Comparatively little work of a fundamental nature has been done in attempting to solve problems of protein metabolism in the study of avian nutrition. Mitchell (6) proposed a method for determining the biological value of protein, modified from the method used by Thomas, using the rat as the experimental animal. A more recent discussion of this method is found in a book by Mitchell and Hamilton (10). This method has not previously been modified for use with poultry, partly because of the lack of a satisfactory method for the determination of uric acid in avian excreta. Mitchell (7) calculated the biological value of corn proteins for chickens, but used an assumed value for the digestibility of the nitrogen as a basis for his calculation.

Ackerson, Blish, and Mussehl (1) determined the total endogenous nitrogen metabolism of mature birds without determining the fecal and urinary nitrogen separately. This work was done on individual birds. Later these authors (3) studied the effect of individual variation upon the total endogenous nitrogen metabolism and concluded that it was better to use group averages in studying nitrogen metabolism.

With the method for the determination of uric acid by St. John and Johnson (13) available, a measurement of the so-called "endogenous" and "metabolic" nitrogen elimination (Mitchell, 6, 7, and Mitchell and Hamilton, 10) of growing chicks has been made. These data are essential for the calculation of biological values by the method proposed by Mitchell. Chicks were studied from time of hatching to twelve weeks of age, and the application of these results to a calculation of the biological value of protein in the study of avian nutrition is shown.

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One hundred and fifty S. C. White Leghorn chicks were placed in the batteries described by St. John, Carver, Helphrey, Miller, and Cassel (12) and maintained on the ration, described by them, containing 16 per cent total protein. A group of 20 chicks was selected at intervals of two weeks and placed upon a low nitrogen diet. The chicks were maintained in individual compartments in a separate battery under the controlled conditions previously described by the above authors, and the samples of excreta were collected, preserved, and analyzed by the method described by St. John and Johnson (13). The total nitrogen was determined by the Gunning modification of the Kjeldahl method (sodium sulfate and copper sulfate). The ammonia nitrogen was determined by weighing out 2 grams of the dried sample, adding 5 grams of magnesium oxide and 200 ml. of distilled water and distilling into standard acid at 250 mm. pressure. The temperature of the distilling liquid was about 75 degrees C.

The low nitrogen diet used consisted of starch 63 per cent, sugar 15 per cent, salt mixture 5 per cent, cod liver oil 7 per cent, charcoal 5 per cent, and grit 5 per cent. The per cent of nitrogen in this ration was 0.042.

A careful record of the feed consumption was made and also of the daily weight of each chick throughout the experimental period. A calculation based upon data in the paper by Mitchell, Card and Hamilton (11) shows that the feed consumed by our chicks was sufficient for their energy requirements. The chicks were placed upon the low nitrogen diet for a preliminary period of two days before collection of excreta was begun. The use of charcoal in this diet permitted a judgment of the necessary length of this preliminary period. The primary purpose of the charcoal was to furnish bulk in the diet. It was evident that practically all of the undigested residue from the natural ration upon which the chicks had been maintained had been expelled from the system within less than 24 hours. Therefore a preliminary period of 48 hours gave a sufficient margin of safety within which all of the food nitrogen had been eliminated from the digestive tract of the chicks. This point is discussed again in this paper.

In determining the most suitable procedure to use in this work two lots of chicks (lots three and six) were studied daily. Lot number three was placed on the low nitrogen feed at the age of seven weeks and the daily consumption of feed determined, while each day's excreta was weighed and preserved separately. These chicks were maintained on the low nitrogen feed for twelve days. The data covering this period are presented in Table I.

A number of workers have studied the composition of avian urine, and these results are summarized by Coulson and Hughes (4). A study of their

Table 2 leads one to the conclusion that the data available indicate that approximately 80 per cent of avian urine is composed of uric acid and ammonia nitrogen. Since this is the closest approximation that can be made at present, we have multiplied the sum of the uric acid nitrogen plus ammonia nitrogen by the factor 1.25 (100/80) to calculate the total urinary nitrogen. The amount of fecal nitrogen per day is found by subtracting the total urinary nitrogen from the total nitrogen elimination.

From Table I it is evident that the total weight of dry excreta for lot three progressively decreases from day to day throughout the period up to the last day. After the first day the food intake shows a regular progressive decrease up to the last of the period. The per cent of total nitrogen and of uric acid steadily increases after the first day until the last day of the period. On the other hand when the grams of total and of uric acid nitrogen eliminated per day are calculated, the amount is found to be very nearly constant after the first day. The grams of ammonia nitrogen eliminated per day are not so closely uniform, but the amount is comparatively small so that it has little influence on the total amount of urinary nitrogen. The latter is also about the same in amount from day to day. The fecal nitrogen is comparatively small in amount and rather regularly decreases through the eleven-day period.

Mitchell and Kick (9) assume that metabolic fecal nitrogen is proportional to feed consumption and that the "excretion of fecal nitrogen per kilogram of food consumed on the nitrogen-free ration, . . . measures the excretion of body nitrogen in the feces" on a diet containing nitrogen. Mitchell (8) discusses the justification for this assumption. The weight of fecal nitrogen per kilogram of low nitrogen food intake has been calculated and presented in Table I. There is no consistent trend in the amount eliminated from day to day. The amount of fecal nitrogen eliminated each day per kilogram of body weight does however appear to decrease in the latter part of the period. The urinary nitrogen elimination per kilogram of chick weight shows a slight tendency to increase throughout the eleven-day period.

Checking differences occurring between each of the first three days in the various columns in Table I shows that in many cases there is more difference between the first and second day than between the second and third and subsequent days. This confirms the indication evident from the use of charcoal in the diet and makes it clear that a two-day preliminary period is sufficient to allow the avian system to adjust itself to the endogenous level of nitrogen excretion. In fact it appears from the data and from the evidence based on the use of charcoal in the low nitrogen ration that a one-day preliminary period would probably be sufficient.

TABLE I

ENDOGENOUS AND METABOLIC NITROGEN OF CHICKS BETWEEN SEVEN AND NINE WEEKS OF AGE (Lot 3)

Day	Total weight of chicks av.	Food intake	Total dry excreta	Total N	Uric acid	NH_3 N	Total N	Total uric acid N	Total NH_3 N	Uric acid N+NH_3 N	Urinary N	Fecal N	Fecal N per kg. food intake	Fecal N per kg. weight per day	Urinary N per kg. of chick wt.
	gm.	gm.	gm.	%	%	%	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	5210	623	168.7	2.93	4.03	.118	4.94	2.27	.20	2.47	3.09	1.85	2.97	.355	.59
2	5304	660	115.2	2.39	3.55	.096	2.75	1.36	.11	1.47	1.84	.91	1.38	.172	.35
3	5238	500	105.1	2.55	4.07	.072	2.68	1.43	.08	1.51	1.89	.79	1.58	.151	.36
4	4948	350	94.5	2.98	4.63	.028	2.82	1.46	.03	1.49	1.86	.96	2.74	.194	.38
5	4734	271	73.7	3.74	6.33	.132	2.76	1.56	.10	1.66	2.08	.68	2.51	.144	.44
6	4465	159	62.6	4.38	7.00	.222	2.74	1.46	.14	1.60	2.00	.74	4.65	.166	.45
7	4273	133	49.7	4.97	8.73	.208	2.47	1.45	.10	1.55	1.94	.53	3.88	.124	.45
8	4086	123	46.8	5.84	11.78	.148	2.73	1.84	.07	1.91	2.39	.34	2.76	.083	.58
9	4005	116	42.2	6.18	12.75	.158	2.61	1.79	.07	1.86	2.33	.28	2.41	.070	.57
10	3706	81	38.0	6.46	12.86	.124	2.45	1.63	.05	1.68	2.10	.35	4.32	.094	.57
11	3610	95	27.0	8.09	17.29	.116	2.18	1.56	.03	1.59	1.99	.19	2.00	.053	.55
12	3164	71	29.6	7.95	15.89	.142	2.35	1.57	.04	1.61	2.01	.34	4.79	.107	.64

TABLE II

ENDOGENOUS AND METABOLIC NITROGEN OF CHICKS BETWEEN ELEVEN AND THIRTEEN WEEKS OF AGE (Lot 6)

Day	Total weight of chicks av.	Food intake	Total dry excreta	Total N	Uric acid	NH_3 N	Total N	Total uric acid N	Total NH_3 N	Uric acid N+NH_3 N	Urinary N	Fecal N	Fecal N per kg. food intake	Fecal N per kg. weight per day	Urinary N per kg. of chick weight	
	gm.	gm.	gm.	%	%	%	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
1	13351	1511	459.1	4.03	6.96	.116	18.50	10.65	.53	11.18	13.98	4.52	2.99	.339	1.05	Commercial
2	13578	1684	676.9	4.14	6.90	.096	28.02	15.57	.65	16.22	20.28	7.74	4.60	.570	1.49	ration.
3	13180	1180	389.5	3.48	5.47	.092	13.55	7.10	.36	7.46	9.33	4.22	3.58	.320	.71	
4	13000	1037	205.2	2.97	3.66	.092	6.09	2.50	.19	2.69	3.36	2.73	2.63	.210	.26	Low
5	12621	1095	300.3	2.38	2.90	.060	7.15	2.90	.18	3.08	3.84	3.30	3.01	.261	.31	
6	12247	644	183.8	3.73	4.45	.096	6.86	2.73	.18	2.91	3.64	3.22	5.00	.263	.30	nitrogen
7	11976	252	227.0	3.09	4.63	.074	7.01	3.50	.17	3.67	4.59	2.42	9.60	.202	.38	
8	11320	491	163.7	3.79	6.20	.144	6.20	3.38	.24	3.62	4.53	1.67	3.40	.148	.40	
9	10808	362	112.7	5.52	8.48	.200	6.22	3.19	.23	3.42	4.28	1.94	5.36	.179	.40	ration.
10	10599	317	107.9	5.63	11.27	.248	6.07	4.05	.27	4.32	5.40	.67	2.11	.063	.51	
11	12378	1411	443.8	3.82	5.08	.116	16.95	7.52	.51	8.03	10.04	6.91	4.90	.558	.81	
12	13228	1559	586.8	3.70	5.44	.124	21.71	10.64	.73	11.37	14.21	7.50	4.81	.567	1.07	Commercial
13	13346	1433	655.7	3.50	5.37	.112	22.95	11.74	.73	12.47	15.59	7.36	5.14	.551	1.17	
14	14078	1491	601.3	3.56	5.77	.112	21.41	11.57	.67	12.24	15.30	6.11	4.10	.434	1.09	
15	14738	1905	700.2	3.56	5.60	.096	24.93	13.07	.67	13.74	17.18	7.75	4.07	.526	1.17	ration.
16	15183	1891	735.9	3.70	6.25	.064	27.23	15.33	.47	15.80	19.75	7.48	3.96	.493	1.30	

An additional lot of chicks (lot 6) was started on the experimental ration at the age of eleven weeks. To obtain a more complete picture of the response of the chicks to a low nitrogen diet, it seemed desirable to study the nitrogen elimination on the natural ration for several days preceding and following the feeding of the low nitrogen diet. The excreta were collected daily for two days before the birds were put on the low nitrogen diet, then for the two-day preliminary period, followed by daily collection for a six-day experimental period (making two three-day periods). Finally the birds were again placed on the natural ration containing added dry skim milk at a 16 per cent protein level and samples were collected daily for an additional six days. The data are presented in Table II.

The per cent of total nitrogen in the dry excreta decreases as soon as the chicks are put on the low nitrogen diet and then increases until they are again placed on the natural ration when the percentage again decreases and continues at a fairly constant level. The total nitrogen elimination immediately decreases with the feeding of the low nitrogen diet and continues at about the same level, then quickly increases to about the former level when the chicks are returned to the nitrogen-containing ration. The per cent of uric acid and total uric acid elimination follow practically the same trend as does the total nitrogen. The same may be said of the ammonia and total urinary nitrogen figures and the urinary nitrogen elimination per kilogram of weight. However, the fecal nitrogen elimination continues to decrease throughout the period of low nitrogen feeding before it rises to the previous level. The grams of fecal nitrogen per kilogram of food intake vary from day to day throughout the total sixteen-day period, although there is a slight tendency for the amount during the low nitrogen period to be below the amount for the remainder of the period. The data for the fecal nitrogen elimination per kilogram of body weight follow in trend those for the total fecal nitrogen.

Other lots of chicks were started at the ages of two, four, nine, and ten weeks. In the case of the first two lots, the collection of excreta samples was made in four-day periods with a four-day preliminary period, while for the rest, on the basis of the data presented above, the periods were three days in length, daily samples being preserved separately in the case of lots three and six. The diet for the first two lots was slightly different from that subsequently used. Data for the second half of lot two are incomplete. The data for lots 1, 2, 4, and 5 are not presented in full but a summary of the data for all six lots is given in Table III. The figures given in this table for lots three and six are averages of the results for each day of the two three-day periods following the preliminary period. The first

and second halves of the period for each lot are tabulated separately in Table III. The fecal nitrogen both per unit of food and per unit of weight are given. The total endogenous nitrogen elimination is also given.

The fecal nitrogen per kilogram of feed intake varies between lots of birds but there is no trend either in the first or second half of the period.

TABLE III

FECAL, URINARY, AND TOTAL NITROGEN ELIMINATION OF CHICKS AT DIFFERENT AGES ON A LOW NITROGEN RATION

Lot	Age at begin. of period	Length of period	Fecal N per kg. food intake	Fecal N per kg. weight per day	Urinary N per kg. weight per day	Total N elimination per kg. per day
No.	weeks	days	gm.	gm.	gm.	gm.
			(First half of period)			
1	2	4	3.30	.149	.52	.669
2	4	4	1.29	.048	.49	.538
3	7	3	2.28	.163	.39	.553
4	9	3	4.07	.253	.33	.583
5	10	3	2.15	.139	.37	.509
6	11	3	4.00	.242	.33	.572
Av. of all lots			2.85*	.166	.405	.57
			(Second half of period)			
1	2	4	2.72	.068	.63	.698
3	7	3	3.80	.124	.49	.614
4	9	3	4.09	.135	.42	.555
5	10	3	3.21	.099	.65	.749
6	11	3	3.62	.130	.44	.570
Av. of all lots			3.49	.111	.526	.637
Av. all lots, both halves			3.14	.141	.460	.601

* Calculating this value to a dry matter basis gives 3.06 grams of fecal nitrogen per kilo. of dry matter in the food intake. There was 6.81 per cent of moisture in the low nitrogen diet.

Mitchell (6) found "unaccountable variations" in the amount of metabolic fecal nitrogen eliminated by rats when this value was determined at successive separated intervals. The average for the second half of the period is larger than that for the first half, but on comparing the first and second half of the period for each lot, it is questionable if this higher average is significant. If the low value for lot two in the first half is omitted, the

average for the five remaining lots is 3.16 grams. The fecal nitrogen per kilogram of body weight appears to be significantly lower in the second half of the periods since not only the average is lower, but the second half is lower for each lot than the first half. Similarly with the urinary nitrogen it appears higher in the second half both in average and for each lot. In none of the data presented in Table III does it appear that there is a variation of either fecal or urinary nitrogen due to differences in the age of the chicks within the twelve-week period covered by this work. Since it would be expected that the chicks would be more nearly normal in the first part of a low nitrogen feeding period than in the latter part, and in view of the facts stated above, the averages for the first half have been accepted for use in the calculation of biological values.

A few determinations of total nitrogen elimination (endogenous plus metabolic nitrogen) have been made on birds from $4\frac{1}{2}$ months to 2 years of age by Ackerson, Blish, and Mussehl (1, 2, 3). Their results vary from 325 to 115 mg. per kilo. per day depending upon the age of the birds. The total nitrogen excreted on a low nitrogen diet decreased as the birds aged. The results for total nitrogen eliminated reported in Table III above, average 570 mg. per kilo. for the first half of the experimental periods. This is 75 per cent above the highest result reported by Ackerson, Blish, and Mussehl and extends the curve for variation with age which their data suggest. They did not determine uric acid and therefore do not report data for endogenous and metabolic nitrogen separately. Also the results for urinary nitrogen reported in Table III above are within the range found by Helphrey (5).

Mitchell (6) states that "rats will ordinarily excrete from 1.5 to 3.0 mg. of nitrogen per gram of nitrogen-free ration consumed." From the context it is understood that he means "metabolic" nitrogen. This agrees closely with the results for chicks reported in Table III. Mitchell gives the amount of urinary nitrogen on a low nitrogen ration in his tables 14 and 15. An average of these data shows 0.212 grams of urinary nitrogen per kilo. of body weight. Judging by weight, the rats used by Mitchell were between four and twelve weeks of age. The average value of 405 mg. of urinary nitrogen per kilo. of body weight shown in Table III above, for chicks, is approximately twice the result obtained by Mitchell for rats.

As an example of the method of calculation of biological values, the data for the fourteenth day of lot 6 (Table II) is used. There were twenty chicks in the lot. The nitrogen intake is calculated from the food intake and in this case is 38.32 (1491×0.0257) grams for the twenty chicks, for this day. The total urinary and fecal nitrogen are given in Table II. The

metabolic nitrogen elimination is computed from the food intake and the average metabolic nitrogen level given in Table III ($1.491 \times 2.85 = 4.25$) while the endogenous nitrogen elimination is determined from the total weight of the twenty chicks and the average endogenous nitrogen per kilogram per day given in Table III ($14.078 \times 0.405 = 5.70$). We then have,

	grams
a = N intake ($1491 \times .0257$)	38.32
b = Total N elimination	21.41
c = Total Urinary N	15.30
d = Fecal N (b–c)	6.11
e = Metabolic N (1.491×2.85)	4.25
f = Food N in feces (d – e)	1.86
g = Endogenous N ($14.078 \times .405$)	5.70
h = Food N in urine (c – g)	9.60

The twenty chicks then received daily $38.32 - 1.86 = 36.46$ grams of absorbed nitrogen. The food nitrogen found in the urine which was wasted in metabolism is the difference between the total urinary nitrogen elimination and endogenous nitrogen and in this case equals $15.30 - 5.70 = 9.60$ grams. It is then evident that $36.46 - 9.60 = 26.86$ grams of nitrogen were retained in the body. Since the biological value as defined by Karl Thomas and by Mitchell is the per cent of absorbed nitrogen which is retained in the body, it is equal in this instance to $100\ (26.86 \div 36.46) = 73.67$. Reduced to a formula the biological value

$$100 \times \frac{[a - (\{b - c\} - e)] - (c - g)}{[a - (\{b - c\} - e)]} =$$

$$100 \times \frac{[38.32 - (\{21.41 - 15.30\} - 4.25)] - (15.30 - 5.70)}{[38.32 - (\{21.41 - 15.30\} - 4.25)]} = 73.67$$

If the value for the metabolic nitrogen per kilogram of chick weight (0.166) is used in place of the method of calculation used above, the biological value is found to be 72.21.

Summary

The endogenous and metabolic nitrogen elimination of S.C. White Leghorn chicks has been studied from time of hatching to twelve weeks of age. Twenty chicks were used in each lot studied. The urinary nitrogen was calculated from the sum of the uric acid nitrogen and ammonia nitrogen elimination by multiplying by 1.25. The per cent of total nitrogen and

uric acid eliminated varies from day to day but the total amount per day is nearly the same throughout the period. The total fecal nitrogen is comparatively small in amount and decreases somewhat during the nitrogen-free period. The fecal nitrogen per kilogram of food intake varies from day to day, but remains at about the same level throughout the period. Calculated per kilogram of body weight, the fecal nitrogen decreases in the latter part of the period. The response of chicks to a low nitrogen diet and to the resumption of a commercial ration is shown.

Within the twelve-week period following hatching no variation of either fecal or urinary nitrogen was found which correlated with the age of the chicks. The averages for the six lots were accepted for use in calculating biological values.

A method of calculating the biological value of protein for use with poultry is presented.

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THE UTILIZATION BY HUMAN SUBJECTS OF THE NITROGEN, CALCIUM, AND PHOSPHORUS OF THE NAVY BEAN (PHASEOLUS VULGARIS) WITH AND WITHOUT A SUPPLEMENT OF CYSTINE*

By Martha S. Pittman

(From the Nutrition Laboratory of the Department of Home Economics and Household Administration of the University of Chicago, Chicago, Ill.)

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Prausnitz (14) reported the results of a digestion experiment on one man who ate daily for 3 days a diet consisting of 500 grams of navy beans and a liter of beer. Since only about 70 per cent of the beans were digested by his subject, Prausnitz concluded that they were not used economically by the body and recommended moderation in employing them in the diet.

A further attempt was made by Snyder (24) to determine the nutritive value of navy beans. He also studied the results of removal of the skins, of cooking the beans with and without the addition of baking soda, and particularly their effect upon the digestibility of other foods. The beans were baked and fed to active young men. For one set of experiments they were eaten with a simple diet of bread and milk, some of the subjects consuming as much as 500 grams of beans daily. The coefficients of digestibility for the protein on this diet appeared to vary greatly with individuals, ranging from 76 to 87 per cent with an average of 80 per cent. With the addition of butter or oleo to the diet the coefficients were higher. If the skins were not removed, 25 per cent less protein was digested and the loss was still greater if the soda were omitted in the cooking process. As a result of these experiments, Snyder also recommended a moderate use of beans in the diet—not to exceed 120 grams of raw beans daily—and stressed the value of proper combinations and preparation.

Wait (25) performed similar experiments using young men as subjects. Besides navy beans, his diets included pork for seasoning, bread, butter, bananas, and sugar. He wished to approximate a mixed diet believing it to be more favorable to digestion. When beans were used in quantities ranging from 375 to 438 grams daily, Wait obtained coefficients of digesti-

* The experimental data in this paper are taken from a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Ogden Graduate School of Science in the University of Chicago, 1930.

bility of 73 to 78 per cent for the protein. With only 150 to 175 grams daily, the coefficients were slightly higher, varying from 78 to 81 per cent. He therefore agreed with the previous workers as to the amounts to be eaten and suggested that beans, as he used them, are less completely digested than other common foods.

The more recent literature on the subject deals largely with animal experiments. McCollum, Simmonds, and Pitz (7) found that when beans were used as the sole source of protein for rats, they appeared to be of low nutritive value resulting in stunted growth and a high mortality rate. These authors therefore recommend that navy beans be supplemented by other proteins of better quality and, because of digestive disturbances, that they be used in moderation.

Johns and Finks (4) were unable to obtain good growth in rats with navy beans until the latter were cooked and cystine was added in the proportion of 2 per cent of the protein by weight. They suggest that heating with water may cause some molecular rearrangement which accounts for the greater digestibility or that it may destroy some toxic quality.

Mitchell (9) obtained with rats at a 10 per cent level of intake, the low biological value of 38.4 per cent for the protein of navy beans after steam cooking. He defines the term "biological value" as the percentage of absorbed nitrogen that is not eliminated in the urine. In similar experiments with other foods at this level, milk proteins averaged 84.7 per cent; potato proteins, 66.7 per cent; oat proteins, 64.9 per cent; and corn proteins, 59.6 per cent.

The possible effect of crude fiber on the excretion of nitrogen, calcium, and phosphorus has been recognized for some time. Since navy beans are relatively high in fiber, this point is to be considered when they play an important part in the diet. Mitchell (10) working with rats which received varying amounts of filter paper in a protein-free diet, found fecal nitrogen increased as much as 42 per cent with this type of roughage. Willard, Whitacre, and Blunt (26) comparing results of feeding human subjects two different diets—one apparently higher in fiber than the other—noted lower coefficients of digestibility for the nitrogen in the high-fiber diet.

In contrast to this view, Sjollema (23) observed that increases in indigestible fiber in the diet of rabbits had little effect on nitrogen output. However, the increased fiber did cause a higher fecal loss of calcium and, to a lesser degree, of phosphorus.

Another factor affecting the usage of calcium and phosphorus may be the amount of carbohydrate in the diet. Bergeim (1) reported, as a result of experiments with rats, that when lactose furnished from 25 to 50 per

cent of the calories the absorption of calcium and phosphorus was improved. This was particularly true of calcium. Such carbohydrates as glucose, maltose, and starch, had little or no effect. Bergeim attributed the superiority of lactose to its tendency to form lactic acid in the alimentary tract which is believed to increase the solubility of calcium phosphate. Earlier work indicated that large quantities of glucose and sucrose taken in concentrated form depressed hydrochloric acid secretion making conditions unfavorable to the absorption of calcium and phosphorus.

Experimental Procedure

Because of the unfavorable reports concerning navy beans for human food, an attempt was made to determine their efficiency as the chief source of nitrogen in a diet providing the essentials for nutrition as recognized today. In doing this the conditions were also unusually favorable for observing the utilization of the calcium and phosphorus. The beans were prepared in two different ways and supplemented with cystine in some cases to determine if the addition of this amino acid, which is considered the chief limiting factor in navy beans, would increase their utilization.

The experiment was divided into two main parts. In the first series of investigations the beans were eaten as a thick purée; in the second, they were baked. These will be designated respectively as the Bean Purée and the Baked Bean Series. Each of these was subdivided as follows:

Without added cystine	
Preliminary period	3 days
Collection period	10 days (2 periods of 5 days each)
With added cystine	
Preliminary period	3 days
Collection period	10 days (2 periods of 5 days each)

Subjects. Five healthy adult women served as subjects, three being used for each phase of the investigation. All were university students engaged in somewhat similar activities involving more or less laboratory and class room work.

Diet. The diet consisted of navy beans, purified butter fat, sucrose, lactose, grape juice, and lemon juice. The quantities used are shown in Table I. Except for Vitamin D it was doubtless adequate unless the quality of protein should prove to be poor.

The protein was reckoned on the basis of 80 per cent of the requirement set by Sherman of 44.4 grams per 70 kilograms of body weight. This low

level was used to permit differences due to the factors under investigation to be detected readily. The beans were used in sufficient quantity to supply from 90 to 94 per cent of the nitrogen, 80 to 85 per cent of the calcium, and from 84 to 95 per cent of the phosphorus of the various diets. The desired number of calories for the different subjects was secured by varying the amounts of fat and sugars consumed.

TABLE I

AMOUNT OF FOOD EATEN BY THE DIFFERENT SUBJECTS

Food	Subject			Subject		
	A	B	C	C	D	E
	gm.	gm.	gm.	gm.	gm.	gm.
Beans, dry	147.5	119.5	138.0	113.8	163.1	120.9
Butter fat	55.5	38.9	44.4	55.5	99.9	55.5
Sucrose	106.3	106.3	81.3	100.0	112.5	100.0
Lactose	68.5	50.0	50.0	50.0	75.9	50.0
Grape juice	600.0	600.0	600.0	600.0	600.0	600.0
Lemon juice	128.2	128.2	128.2	128.2	128.2	128.2

TABLE II

DIET AS CALCULATED FOR THE VARIOUS SUBJECTS

Subject	Protein	Calcium	Phosphorus	Calories per kg.	Total calories	Excess N. base*
	gm.	gm.	gm.			cc.
Bean purée series						
A	33.2	0.333	0.774	36.0	2358	55.22
B	26.9	0.288	0.642	38.0	2037	50.17
C	31.0	0.318	0.729	37.7	2051	53.50
Baked bean series						
C	25.6	0.279	0.615	42.4	2143	49.14
D	36.7	0.358	0.847	39.6	2863	58.02
E	27.2	0.290	0.648	40.4	2167	50.42

* Without added cystine.

Distilled water was used for all purposes. It was taken *ad libitum* during the Bean Purée Series and the amount consumed varied considerably with the individual and the weather. In the Baked Bean Series each subject took approximately 1200 cc. daily in addition to that obtained in the food. This included a small cup of black coffee for two of the subjects each morning.

In the latter half of each series, l-cystine, in the proportion of 2 per cent of the weight of the calculated protein, was added to the diet. The allowance for each day was sprinkled over the beans at the time of eating. The amount of salt used (C. P. grade) was not controlled but in no case was it excessive.

The beans were purchased in two lots, one for each series of experiments. The day's supply for each individual was prepared separately on the day before it was to be used. After soaking over night, the beans for purée were salted, and brought to the boiling point over the direct heat, after which they were covered and cooked until soft. This required from 4 to 5 hours in a regulated oven set at 250° F. They were then run through a sieve and all pulp and washings returned as completely as possible, so the loss in preparation was negligible. They were divided into 3 portions, one for each meal. The butter fat was added when they were reheated for serving.

The beans for baking, after soaking, were put into individual baking dishes, one for each meal, and baked with the salt at 350 to 375° F. for 10 hours. The butter fat was added for the last 30 minutes of baking.

The remainder of the diet was combined into a fruit punch. The three meals a day therefore consisted merely of beans and punch.

The amount of roughage in the diet was limited to that present in the beans. The acid-base balance was the same for both periods without added cystine. The excess base was unusually high and, even after cystine was added, the diet was still strongly basic.

The calories remained the same for each subject in both series except for the small increase due to the cystine in the latter half of each period. Because of some loss of weight by the subjects during the Bean Purée Series, which averaged 36 to 38 calories per kilogram, the calories were increased in the Baked Bean Series ranging from 39.6 to 42.4 per kilogram for the various subjects.

Samples. The usual precautions were observed in the collection and handling of samples. Aliquot portions of the foods were saved each day. The beans were cooked, dried at 80° C., ground, and thoroughly mixed. The fruit juices were combined and preserved by canning by the cold-pack method using a water bath. Butter fat, sucrose, and lactose were not sampled as they were considered 100 per cent pure and therefore no analyses were thought necessary.

The urine was preserved with toluol and hydrochloric acid. Each day's collection was kept separate until after nitrogen determinations were made. The feces were mixed with acidified alcohol before drying. A composite was made for each 5-day period.

Methods for Analysis. The pH of the urine was obtained colorimetrically. Total nitrogen was determined by the Kjeldahl-Gunning procedure. Calcium was obtained volumetrically by a modified McCrudden method (8) using Shohl's (20) and Shohl and Pedley's (21) suggestions for controlling pH. Total phosphorus was determined gravimetrically by the Neumann method (12) as modified by Lundell and Hoffman (5) and McCandless and Burton (6). Accuracy of technic was proven by analysis of materials of known composition.

Results and Discussion

The subjects maintained good health throughout the experiment with the exception that mild indigestion, apparently due to formation of gas, was reported by two subjects in the Bean Purée Series. This difficulty disappeared after the first few days. Since one explanation for such gas is the action of bacteria on fiber, more trouble was expected in the Baked Bean Series in which the fiber was not finely divided. However no trouble occurred, perhaps because of the longer cooking period of the baked beans or the fact that the purée was eaten in hot weather when beans are less appetizing.

It was believed the diet supplied an abundance of calories, yet a slight loss of weight occurred with all subjects. It is possible that with the high-fiber diet used in this experiment less food was absorbed than was expected, making the calories insufficient. However, increased calories in the Baked Bean Series did not decrease the loss in weight, so some other factor was doubtless involved.

The diet was deficient in Vitamin D but experimental work indicates that lack of this vitamin in the diet of normal adults for so brief a time as that covered by this investigation would not affect the results materially.

Urine Findings. The pH of the urine varied greatly with individuals and also in the same individual from day to day even though the diet was practically constant in every respect. The urine seemed to increase in acidity when the weather was warm. The addition of cystine lowered the potential alkalinity of the diet but it did not always result in a more acid urine.

Although some exceptions occurred, the average pH of the urine was more acid than the 6.64 suggested by Hawk and Bergeim (3) for a vegetable diet. The explanation may be, as offered by Pickens and Hetler (13), that although the basic elements are high in grape juice they do not increase the alkalinity of the urine as expected.

Feces Findings. The daily wet weight for the feces ranged from 64.3 to

174.7 grams a day. The average for all periods and subjects was 123.7 grams a day which was equivalent to 25.9 grams dry weight. The average amount of residue, with some exceptions, was not so high as was anticipated because of the fiber in the beans. In accordance with expectations, it was slightly lower in the Bean Purée Series where the beans were broken by running through a sieve.

Nitrogen Findings. In these experiments no corrections were made for endogenous nitrogen. However, the results appearing in Tables III and IV are believed to indicate the efficiency of the navy bean as a source of nitrogen as used in these experiments.

The loss of nitrogen in the feces for both series of experiments ranged from 23 to 43 per cent with an average of approximately 34 per cent. In the Bean Purée Series the excretion of nitrogen in the feces varied from 1.10 to 2.71 grams with an average for all subjects of 1.87 grams. In the Baked Bean Series the excretion ranged from 1.45 to 2.41 grams and averaged 1.93 grams. It is thus seen that the loss was practically the same for both methods of cooking. The large amount of fiber in the beans probably explains such high fecal nitrogen on a diet averaging only 5.2 grams of nitrogen per capita per day.

The average coefficient of digestibility ranged from 57 to 77 per cent for the the various subjects. Both of these extremes were obtained on the Bean Purée diet. The average coefficient of digestibility was 65.9 per cent for all subjects for both periods, so no difference can be attributed to the method of cooking.

In contrast to this, Wait (25) reported an average coefficient of 78 per cent for the nitrogen of white beans used in a mixed diet while Mitchell (9) obtained for rats on a bean diet a value of 76 per cent after correction had been made for metabolic nitrogen in the feces. The lower coefficients obtained in this investigation probably are explained to a large extent by the high crude fiber content of the diet. The fact that the beans were eaten in fairly large quantities, furnishing approximately 20 per cent of the calories consumed, may also have been a factor. It may explain too, a somewhat similar value of 70 per cent obtained by Prausnitz (14) when his subject subsisted entirely upon beans and beer. This harmonizes also with Snyder's higher figures of 80 per cent when some milk was used and of 88 to 90 per cent when a greater variety of foods was eaten.

It appears that the nitrogen from the beans, supplemented by the very small quantities obtained from the remainder of the diet, was inadequate to maintain the body in nitrogen equilibrium for any length of time. The nitrogen intake ranged from 4.23 to 6.73 grams per day or 0.082 to 0.093

TABLE III
NITROGEN FINDINGS
Bean Purée Series

Subject	Expt.	Period	Average daily intake				Average daily output					Balance
			Beans	Other foods	Cystine	Total	Urine	Feces	Total	Urine	Feces	
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	gm.
A	Non-cystine	I	4.71	0.41	—	5.12	3.81	1.30	5.11	74.6	25.4	+0.01
		II	4.91	0.40	—	5.31	3.53	2.16	5.69	62.0	38.0	−0.38
		Av.	4.81	0.41	—	5.22	3.67	1.73	5.40	68.3	31.7	−0.19
	Cystine	III	5.06	0.38	0.08	5.52	3.19	1.60	4.79	66.6	33.4	+0.73
		IV	5.10	0.38	0.08	5.56	3.94	1.85	5.79	68.0	32.0	−0.23
		Av.	5.08	0.38	0.08	5.54	3.57	1.73	5.29	67.3	32.7	+0.25
B	Non-cystine	I	3.82	0.41	—	4.23	3.68	1.10	4.78	77.0	23.0	−0.55
		II	3.98	0.40	—	4.38	3.67	1.75	5.42	67.7	32.3	−1.04
		Av.	3.90	0.41	—	4.31	3.68	1.43	5.10	72.4	27.7	−0.80
	Cystine	III	4.10	0.38	0.06	4.54	3.82	1.60	5.42	70.5	29.5	−0.88
		IV	4.13	0.38	0.06	4.57	3.35	2.03	5.38	66.3	33.7	−0.81
		Av.	4.12	0.38	0.06	4.56	3.59	1.82	5.40	68.4	31.6	−0.85
C	Non-cystine	I	4.41	0.41	—	4.82	3.60	2.71	6.31	57.0	43.0	−1.49
		II	4.59	0.40	—	4.99	3.53	2.25	5.78	61.1	38.9	−0.79
		Av.	4.50	0.41	—	4.91	3.57	2.48	6.05	59.1	41.0	−1.14
	Cystine	III	4.73	0.38	0.07	5.18	3.27	2.06	5.33	61.4	38.6	−0.15
		IV	4.77	0.38	0.07	5.22	3.05	2.03	5.08	60.1	39.9	+0.14
		Av.	4.75	0.38	0.07	5.20	3.16	2.05	5.21	60.8	39.3	−0.01

TABLE IV
NITROGEN FINDINGS
Baked Bean Series

Subject	Expt.	Period	Average daily intake				Average daily output					Balance
			Beans	Other foods	Cystine	Total	Urine	Feces	Total	Urine	Feces	
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	gm.
C	Non-cystine	V	4.23	0.35	—	4.58	3.49	1.96	5.45	64.0	36.0	−0.87
		VI	4.36	0.35	—	4.71	3.14	2.10	5.24	59.9	40.1	−0.53
		Av.	4.30	0.35	—	4.65	3.32	2.03	5.35	62.0	38.1	−0.70
	Cystine	VII	4.38	0.37	0.06	4.81	3.30	1.92	5.22	63.2	36.8	−0.41
		VIII	4.33	0.36	0.06	4.75	3.10	1.93	5.03	61.1	38.9	−0.28
		Av.	4.36	0.37	0.06	4.78	3.20	1.93	5.13	62.2	37.9	−0.35
D	Non-cystine	V	6.07	0.35	—	6.42	5.14	2.13	7.27	70.7	29.3	−0.85
		VI	6.24	0.35	—	6.59	4.75	2.41	7.16	66.3	33.7	−0.57
		Av.	6.16	0.35	—	6.51	4.95	2.27	7.22	68.5	31.5	−0.71
	Cystine	VII	6.28	0.37	0.08	6.73	4.79	1.85	6.64	72.2	27.8	+0.09
		VIII	6.18	0.36	0.08	6.62	4.25	1.67	5.92	71.8	28.2	+0.70
		Av.	6.23	0.37	0.08	6.68	4.52	1.76	6.28	72.0	28.0	+0.40
E	Non-cystine	V	4.50	0.35	—	4.85	3.30	1.89	5.19	63.5	36.5	−0.34
		VI	4.63	0.35	—	4.98	3.69	1.45	5.14	71.8	28.2	−0.16
		Av.	4.57	0.35	—	4.92	3.50	1.67	5.17	67.7	32.4	−0.25
	Cystine	VII	4.64	0.37	0.06	5.07	3.49	2.32	5.81	60.1	39.9	−0.74
		VIII	4.59	0.36	0.06	5.01	3.00	1.54	4.54	66.1	33.9	+0.47
		Av.	4.62	0.37	0.06	5.04	3.25	1.93	5.18	63.1	36.9	−0.14

grams per kilogram. When furnished by such foods as milk, bread and milk, and meat, this amount of nitrogen was found by Rose and MacLeod (17) to be sufficient to maintain a generous positive nitrogen balance.

The results of addition of cystine were not striking. However, all subjects responded favorably to it in the Baked Bean Series and two of the three subjects in the Bean Purée Series. The range for the nitrogen balance of the subjects on the non-cystine diet averaged −1.14 to −0.19 grams for the different periods, whereas with cystine it ranged from −0.35 to +0.40 grams. In two cases the average balance became slightly positive; one subject was in approximate equilibrium, and two were somewhat less negative. In the remaining instance, the difference between the non-cystine and cystine periods was negligible, the balance changing only from −0.80 to −0.85 grams.

In three subjects the improvement took place parallel with an increase of nitrogen in the feces which indicated that in these instances, at least, the higher coefficients were not associated with better nitrogen absorption. This work confirmed that of the earlier investigators to the extent that the nitrogen of the beans, prepared as described in these experiments, apparently was not well utilized by the body.

Similarly, Sherman and Winters (18) and Sherman, Winters, and Phillips (19) were unable to secure nitrogen equilibrium on diets in which corn or oats furnished most of the protein of the diet. These results thus appeared to support the generally prevalent idea that vegetable proteins are less adequate, used alone, than those from animal sources. Snyder's statement (24) that navy beans may be eaten in comfort in amounts of 4 ounces or more (dry weight) a day is also confirmed. Further, the findings in this experiment agreed with those of Mitchell (11) and McCollum (7) that navy beans cannot be regarded as a "meat substitute" when used as the chief source of nitrogen in the diet. However, it appeared probable that a diet consisting largely of navy beans as a source of nitrogen may be made adequate with the addition of a sufficient amount of supplementary protein or cystine.

Calcium Findings. Tables V and VI show that the subjects were practically all in slightly negative calcium balance throughout the experiment and there was little tendency to acquire equilibrium as the diet was continued. Cystine apparently had no favorable effect on calcium usage since all subjects but one showed an increasingly negative average balance after it was added to the diet. The utilization of calcium seemed to be about equal for the two methods of cooking, the Bean Purée Series having a slight advantage.

TABLE V
CALCIUM FINDINGS
Bean Purée Series

Subject	Expt.	Period	Average daily intake			Average daily output					Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces	
			gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	gm.
A	Non-cystine	I	0.287	0.060	0.347	0.042	0.286	0.328	12.9	87.1	+0.019
		II	0.314	0.061	0.375	0.053	0.538	0.591	9.0	91.0	−0.216
		Av.	0.301	0.061	0.361	0.048	0.412	0.460	11.0	89.1	−0.099
	Cystine	III	0.304	0.061	0.365	0.086	0.460	0.546	16.0	84.0	−0.181
		IV	0.324	0.058	0.382	0.080	0.468	0.548	14.9	85.1	−0.166
		Av.	0.314	0.060	0.374	0.083	0.464	0.547	15.5	84.6	−0.174
B	Non-cystine	I	0.233	0.060	0.293	0.032	0.226	0.258	10.6	89.4	+0.035
		II	0.254	0.061	0.315	0.041	0.382	0.423	9.6	90.4	−0.108
		Av.	0.244	0.061	0.304	0.037	0.304	0.341	10.1	89.9	−0.037
	Cystine	III	0.246	0.061	0.307	0.056	0.362	0.418	13.4	86.6	−0.111
		IV	0.263	0.058	0.321	0.067	0.442	0.509	13.2	86.8	−0.188
		Av.	0.255	0.060	0.314	0.062	0.402	0.464	13.3	86.7	−0.150
C	Non-cystine	I	0.268	0.060	0.328	0.067	0.317	0.384	18.1	81.9	−0.056
		II	0.293	0.061	0.354	0.085	0.391	0.476	17.8	82.2	−0.122
		Av.	0.281	0.061	0.341	0.076	0.354	0.430	18.0	82.1	−0.089
	Cystine	III	0.284	0.061	0.345	0.127	0.351	0.478	26.5	73.5	−0.133
		IV	0.303	0.058	0.361	0.120	0.334	0.454	26.4	73.6	−0.093
		Av.	0.294	0.060	0.353	0.124	0.343	0.466	26.5	73.6	−0.113

TABLE VI
CALCIUM FINDINGS
Baked Bean Series

Subject	Expt.	Period	Average daily intake			Average daily output					Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces	
			gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	gm.
C	Non-cystine	V	0.255	0.066	0.321	0.094	0.350	0.444	21.2	78.8	−0.123
		VI	0.281	0.065	0.346	0.119	0.367	0.486	24.5	75.5	−0.140
		Av.	0.268	0.066	0.334	0.107	0.359	0.465	22.8	77.2	−0.132
	Cystine	VII	0.254	0.072	0.326	0.104	0.344	0.448	23.2	76.8	−0.122
		VIII	0.256	0.065	0.321	0.101	0.380	0.481	21.0	79.0	−0.160
		Av.	0.255	0.069	0.324	0.103	0.362	0.465	22.1	77.9	−0.141
D	Non-cystine	V	0.366	0.066	0.432	0.068	0.441	0.509	13.4	86.6	−0.077
		VI	0.403	0.065	0.468	0.105	0.530	0.635	16.5	83.5	−0.167
		Av.	0.385	0.066	0.450	0.087	0.486	0.573	15.0	85.0	−0.122
	Cystine	VII	0.364	0.072	0.436	0.095	0.491	0.586	16.2	83.8	−0.150
		VIII	0.367	0.065	0.432	0.106	0.405	0.511	20.7	79.3	−0.079
		Av.	0.366	0.069	0.434	0.101	0.448	0.549	18.4	81.6	−0.115
E	Non-cystine	V	0.271	0.066	0.337	0.029	0.419	0.448	6.5	93.5	−0.111
		VI	0.299	0.065	0.364	0.035	0.387	0.422	8.3	91.7	−0.058
		Av.	0.285	0.066	0.351	0.032	0.403	0.435	7.4	92.6	−0.085
	Cystine	VII	0.270	0.072	0.342	0.030	0.537	0.567	5.3	94.7	−0.225
		VIII	0.272	0.065	0.337	0.037	0.433	0.470	7.9	92.1	−0.133
		Av.	0.271	0.069	0.340	0.034	0.485	0.519	6.6	93.4	−0.179

The urinary calcium was low averaging 15.6 per cent of the entire output. Fecal excretion was correspondingly high.

The results for the bean calcium were scarcely so favorable as those obtained by Rose (15), Blatherwick and Long (2), and other workers with calcium from vegetable sources. They resembled those of Rose and MacLeod (17) where almonds supplied from 85 to 86 per cent of the calcium of the diet. These workers were unable to obtain calcium equilibrium on so large a proportion of almonds until the calcium per kilogram of body weight was increased from 8 to 12 milligrams, whereas with other vegetable foods and with a smaller per cent of almond calcium, from 5.6 to 7.0 milligrams of calcium per kilogram of body weight were ample. The diet used in these experiments furnished from 5.5 to 6.5 milligrams of calcium per kilogram of body weight of which 80 to 85 per cent was obtained from the beans.

While it must be granted that the loss of calcium was not great and that the diet provided a scant margin of safety, there seemed to be little tendency to reach a state of equilibrium on the diet. Apparently this amount of calcium, when supplied largely by beans, was insufficient for maintenance. It is probable that the difference was not in the calcium itself but was due to its association with indigestible material.

Phosphorus Findings. The average for each period for each subject as seen in Tables VII and VIII showed a slightly negative phosphorus balance. In four out of six cases the negativity decreased during the cystine periods. When the utilization of the nitrogen improved, with two exceptions, that of the phosphorus improved also. The method of cooking apparently was not an important factor.

In five cases the greater part of the phosphorus was excreted through the feces but the differences were less marked than with calcium. The average loss in the urine was approximately 40 per cent, the remaining 60 per cent being excreted in the feces. In all cases the fecal phosphorus increased during the cystine periods but in only three instances were the differences sufficient to appear significant. Whether this increase of fecal phosphorus meant a poorer digestion of the food, it is impossible to state, but in two out of these three cases there was a parallel decrease in fecal nitrogen indicating better absorption of this element at least.

The average phosphorus intake was low, ranging from an average of 0.551 to 0.769 grams per day or 10.3 to 11.5 milligrams per kilogram of body weight. It is doubtful whether equilibrium could be expected with so small an amount in the food. Certainly the quantities used, when supplied by navy beans, were inadequate to maintain the body in phosphorus equi-

TABLE VII
PHOSPHORUS FINDINGS
Bean Purée Series

Subject	Expt.	Period	Average daily intake			Average daily output					Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces	
			gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	gm.
A	Non-cystine	I	0.554	0.088	0.642	0.245	0.390	0.635	38.6	61.4	+0.007
		II	0.583	0.092	0.675	0.336	0.596	0.932	49.2	50.8	−0.257
		Av.	0.569	0.090	0.659	0.291	0.493	0.784	43.9	56.1	−0.125
	Cystine	III	0.572	0.088	0.660	0.264	0.438	0.702	37.6	62.4	−0.042
		IV	0.583	0.091	0.674	0.246	0.521	0.767	32.1	67.9	−0.093
		Av.	0.578	0.090	0.667	0.255	0.480	0.735	34.9	65.1	−0.068
B	Non-cystine	I	0.449	0.088	0.537	0.335	0.253	0.588	36.0	64.0	−0.051
		II	0.473	0.092	0.565	0.311	0.408	0.719	57.0	43.0	−0.154
		Av.	0.461	0.090	0.551	0.323	0.331	0.654	46.5	53.5	−0.103
	Cystine	III	0.463	0.088	0.551	0.335	0.402	0.737	45.6	54.5	−0.186
		IV	0.470	0.091	0.561	0.330	0.492	0.822	42.8	57.2	−0.261
		Av.	0.467	0.090	0.556	0.333	0.447	0.780	44.2	55.8	−0.224
C	Non-cystine	I	0.518	0.088	0.606	0.368	0.432	0.800	46.0	54.0	−0.194
		II	0.546	0.092	0.638	0.320	0.511	0.832	38.5	61.5	−0.194
		Av.	0.532	0.090	0.622	0.344	0.472	0.816	42.3	57.7	−0.194
	Cystine	III	0.535	0.088	0.623	0.274	0.486	0.760	36.1	63.9	−0.137
		IV	0.545	0.091	0.636	0.249	0.447	0.696	35.8	64.2	−0.060
		Av.	0.540	0.090	0.630	0.262	0.467	0.729	36.0	64.0	−0.099

TABLE VIII
PHOSPHORUS FINDINGS
Baked Bean Series

Subject	Expt.	Period	Average daily intake			Average daily output					Balance
			Beans	Other foods	Total	Urine	Feces	Total	Urine	Feces	
			gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	gm.
C	Non-cystine	V	0.462	0.079	0.541	0.294	0.380	0.674	43.9	56.1	−0.133
		VI	0.485	0.086	0.571	0.251	0.445	0.696	36.1	63.9	−0.125
		Av.	0.474	0.083	0.556	0.273	0.413	0.685	40.0	60.0	−0.129
	Cystine	VII	0.475	0.090	0.565	0.270	0.404	0.674	40.1	59.9	−0.109
		VIII	0.471	0.092	0.563	0.267	0.430	0.697	38.3	61.7	−0.134
		Av.	0.473	0.091	0.564	0.269	0.417	0.686	39.2	60.8	−0.122
D	Non-cystine	V	0.663	0.079	0.742	0.373	0.476	0.849	43.9	56.1	−0.107
		VI	0.694	0.086	0.780	0.359	0.586	0.945	38.0	62.0	−0.165
		Av.	0.679	0.083	0.761	0.366	0.531	0.897	40.9	59.1	−0.136
	Cystine	VII	0.680	0.090	0.770	0.309	0.539	0.848	36.4	63.6	−0.078
		VIII	0.676	0 092	0.768	0.324	0.453	0.777	41.7	58.3	−0.009
		Av.	0.678	0.091	0.769	0.317	0.496	0.813	39.0	61.0	−0.044
E	Non-cystine	V	0.490	0.079	0.569	0.254	0.405	0.659	38.6	61.4	−0.090
		VI	0.515	0.086	0.601	0.235	0.366	0.601	39.1	60.9	±0.000
		Av.	0.503	0.083	0.585	0.245	0.386	0.630	38.8	61.2	−0.045
	Cystine	VII	0.503	0.090	0.593	0.223	0.553	0.776	28.7	71.3	−0.183
		VIII	0.501	0.092	0.593	0.252	0.428	0.680	37.1	62.9	−0.087
		Av.	0.502	0.091	0.593	0.238	0.491	0.728	32.9	67.1	−0.135

librium although some tendency to improved utilization with continued usage was shown by some subjects. This seemed to be associated with somewhat better retention of nitrogen.

The low urinary excretion of both calcium and phosphorus is to be expected because the diet was low in these elements and high in fiber, both of which are believed to increase excretion of these elements by way of the intestine. However, other factors may also be involved.

It is possible, according to Shohl, Bennett, and Weed (22), that the utilization of both the calcium and phosphorus would have been better had the diet been approximately neutral in reaction. On the other hand, the acids of the fruit juices presumably furnished a favorable medium for absorption of both of these minerals by increasing their solubility.

It also is possible that the large amounts of sugar consumed which, including that of the fruit juices, supplied from 50 to 63 per cent of the total calories, may have affected the absorption of the calcium and phosphorus (Bergeim, 1). Approximately 16 to 20 per cent of the sugar calories was supplied by lactose. The benefit that may have been derived from the latter may have been more or less offset by the depressing effects of the other sugars so it is not believed that this factor could have changed materially the results obtained.

Summary

A study was made of the utilization of the nitrogen, calcium, and phosphorus of the navy bean. Five adult women served as subjects. The beans were cooked in two ways—as bean purée and baked beans. They were eaten with and without a supplement of cystine in the proportion of 2 per cent of the weight of the calculated protein.

The diet—consisting of beans, purified butter fat, sucrose, lactose, grape juice, and lemon juice—was presumably adequate except for some deficiency of Vitamin D.

The following tendencies were observed:

The trend of the nitrogen balance was increasingly negative while the beans were eaten without the cystine supplement.

Addition of cystine in the above proportions, although showing some irregularities, apparently improved nitrogen retention slightly.

The calcium and phosphorus of navy beans in the amounts used were, as a rule, unable to maintain the balance of these elements in the body. The phosphorus was utilized somewhat better than the calcium and showed a slightly favorable response to addition of cystine.

Improved utilization of the phosphorus in most cases appeared to parallel better retention of nitrogen. No such correlation was noted between calcium and nitrogen or calcium and phosphorus.

The methods of cooking used apparently had little effect upon the usage of the elements studied.

Navy beans seemingly can be eaten in comfort by healthy people in quantities at least as large as 4 ounces, dry weight, daily.

This work on human subjects appears to support the findings of others that navy beans are not entirely satisfactory as the chief source of nitrogen in the diet.

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THE VALUE OF SOME VEGETABLES IN NUTRITIONAL ANEMIA

By

HAROLD LEVINE, F. B. CULP AND C. B. ANDERSON

(From the Laboratory of the South Carolina Food Research Commission and the Department of Nutrition of the Medical College of the State of South Carolina, Charleston.)

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THE more recent investigations (1 to 4) in the study of nutritional anemia have served to emphasize anew the specificity of copper in supplementing iron in the cure of this form of anemia. During the active controversy as to what mineral elements were concerned in hemoglobin regeneration, the studies were made, for the most part, with solutions of inorganic salts carrying the metals under investigation. As a result, in only a few cases have vegetables been studied in an effort to determine their effectiveness in blood formation. A review of the literature revealed the following studies on the influence of various vegetables when fed in different forms.

Mitchell and Schmidt (5) fed dried spinach to anemic rats and found it effective in the regeneration of hemoglobin. The spinach was fed daily at a level of 1.54 gm. of dried material, furnishing 0.4 mg. of iron. Mitchell and Miller (6) found "a concentrated water extract of dried spinach" effective in blood regeneration in rats. The ash of this extract or an HCl solution of the ash of the extract was also potent in curing anemia.

The Wisconsin workers (7 to 9), in their studies, employed both lettuce ash and an HCl extract of lettuce ash and showed that these sources brought about regeneration of hemoglobin.

Sheets, Frazier and Sulzby (10) studied the effect of feeding dried mustard greens, turnip greens and collards to rats suffering from nutritional anemia. These dried vegetables were found to be potent in hemoglobin regeneration.

From the above resumé of the literature, it is evident that more emphasis has been placed upon the ash than upon the natural food which contains more or less indigestible material. Much needed information is therefore lacking as to the value of various vegetables when consumed in their original or dried state. That the results obtained on the ash of vegetables cannot always be transferred to the natural food is apparent from a study by Bloom (17). This investigator, in a study of the effect of crude fiber on calcium and phosphorus retention in rats, found that the calcium and phosphorus of spinach ash were better utilized than that of either raw or cooked dried spinach.

Since it is possible that the availability of the iron or copper present in the intact or the dried vegetable might be different from that in the

plant ash or in an acid solution of the plant ash, we decided to determine the effectiveness of various dried vegetables when fed to anemic rats in order to ascertain whether or not vegetables can serve as physiologically utilizable sources of these mineral elements. It is believed that the results of such an investigation would be applicable to anemia of nutritional origin in man.

Accordingly the following vegetables—spinach, lettuce, tomato, asparagus, broccoli and turnip greens—were studied for their effectiveness in curing nutritional anemia in the rat. Inasmuch as it was impractical to preserve the same lot of fresh plant tissue for the duration of the experiment, recourse was had to the feeding of the dried ground vegetable. We were particularly interested in turnip greens since this vegetable is a popular salad green in this locality.

Experimental

The technic used in the breeding of rats, the production of nutritional anemia, the drawing of blood samples, the determination of hemoglobin, the analysis of vegetables and milk powder for iron and copper, etc., was the same as described in a previous publication (11) from this laboratory with the exceptions indicated below. In place of using reconstituted whole milk powder[1] to produce anemia, the dried whole milk powder and distilled water[2] were fed, since we could find no apparent differences in the behavior of the rats when the two sources of milk were compared. Palmer and Kennedy (12) found average digestibility values of 95.6 per cent and 92.7 per cent for whole milk powder and liquid milk, respectively. Enough milk powder representing the same lot of liquid milk was purchased so as to last throughout the entire experimental period. Upon analysis, the milk powder was found to contain 3.6 p.p.m. of iron and 1.89 p.p.m. of copper. In the present experiments, the rats were placed on a milk régime as soon as the average body weight of the animals in each litter reached 40 gm. (approximately at 21 days of age) instead of 60 gm. as in our previous experiments (11). This change in procedure reduced the time required to produce a marked anemia from a period of 6 to 8 weeks to a period of 4 to 6 weeks. Smythe and Miller (13) in analyzing the iron content of the bodies of rats at different ages found the lowest iron content at 20 days of age. This finding would seem to explain the ability to develop a severe anemia in our 21-day-old rats in a shorter time.

In determining the effectiveness of various vegetables in anemia, two

[1] Klim.

[2] Containing only negligible amounts of iron and copper.

series of curative experiments were carried out. The duration of the curative period in both series was eight weeks.

Series I

In the first series, which was preliminary in nature, a total of 31 rats was used. In this series, the hemoglobin content of the rats' blood was allowed to drop to an average value of approximately 6.0 gm. per 100 cc. of blood before supplements were fed. Hemoglobin determinations were carried out on a group of twenty-eight rats, representing four litters, reared on our stock ration, and approximately the same age (84 to 89 days) as our experimental rats after about 4 weeks on supplements in the curative period. The range in values found was from 14.0 to 16.8 gm. hemoglobin per 100 cc. of blood (indicated as the "normal zone" in Charts I and II) with an average value of 14.8 gm. Mitchell and Miller (4) report a range in values from 15.3 to 16.6 gm. after their colony rats are 7 weeks of age. The blood of our anemic rats at the start of the curative period contained only 40 per cent of the normal content of hemoglobin.

At the start of the curative period, the animals were separated into groups, placed in individual galvanized iron wire cages and fed various supplements as indicated in Table I. In order to insure a sufficiency of vitamins A and D throughout the curative period, each rat was fed one drop of cod liver oil daily in this and in the following series. Krauss (14) found cod liver oil to be without effect in nutritional anemia in rats. Table I and Chart I serve to show clearly the results obtained. As a means of comparison of the effectiveness of the different supplements, 14.0 gm. of hemoglobin per 100 cc. of blood were chosen as an indication of a fair degree of blood regeneration, this value representing the lower limit in the range of normal hemoglobin values for our stock animals. In the present discussion, therefore, this value will be taken to indicate regeneration of hemoglobin on a given supplement. Mitchell and Miller (6) also used this value for comparative purposes in their anemia experiments on rats.

From a study of Table I and Chart 1, the following facts are borne out. The negative control animals on milk alone showed slightly decreasing hemoglobin values as the curative period progressed. The hemoglobin value for this group averaged 7.2 gm. at the start of the curative period for the reason that animals with the highest blood values were purposely placed in this group. The normal control rats on the stock ration maintained an almost constant hemoglobin value throughout the curative period. These same rats had been on this ration throughout the curative period while the remainder of the animals were being rendered anemic on milk. When

TABLE I

AVERAGE WEEKLY HEMOGLOBIN VALUES* AT TIME SUPPLEMENT WAS ADDED AND AT INTERVALS THEREAFTER, OF ANIMALS RECEIVING VARIOUS AMOUNTS OF IRON AND COPPER IN THE FORM OF DRIED SPINACH OR SOLUTIONS OF INORGANIC SALTS

	Negative controls on milk	Normal controls on stock diet	0.50 Iron	0.20 Copper	0.50 Iron plus 0.050 copper	Dried Spinach			
						Hemoglobin	Average daily spinach intake‖	Average daily iron intake	Average daily copper intake
			mg.	mg.	mg.		gm.	mg.	mg.
At time† of addition	7.2 (3)‡	14.8 (4)	5.9 (9)	6.2 (3)	6.1 (4)	6.0 (8)	—	—	—
	After addition (weeks)								
1	6.6 (3)	14.7 (4)	7.5 (9)	9.3 (3)	11.1 (4)	9.5 (8)	0.51	0.18	0.0026
2	6.2 (3)	15.2 (4)	8.4 (8)§	9.6 (3)	13.5 (4)	11.6 (8)	1.08	0.38	0.0055
3	6.1 (3)	14.9 (4)	10.3 (7)§	9.8 (3)	14.3 (4)	13.1 (8)	1.18	0.43	0.0060
4	5.9 (3)	14.8 (4)	11.4 (6)§	10.6 (2)§	14.7 (4)	14.1 (8)	1.56	0.56	0.0080
5	5.8 (3)	15.4 (4)	10.8 (5)§	9.7 (2)	15.0 (4)	14.8 (8)	1.38	0.49	0.0070
6	5.3 (3)	15.1 (4)	11.7 (5)	9.7 (2)	14.6 (4)	14.9 (8)	1.45	0.52	0.0074
7	4.8 (3)	14.8 (4)	10.4 (5)	10.3 (2)	14.9 (4)	15.2 (8)	1.23	0.44	0.0063
8	5.1 (3)	14.8 (4)	11.8 (5)	9.8 (2)	14.8 (4)	15.0 (8)	1.23	0.44	0.0063
Time (weeks) to reach 14.0 gm. hemoglobin	—	—	—	—	2–3	3–4			

* Expressed as grams of hemoglobin per 100 cc. of blood.

† After 5–6 weeks of preliminary feeding on dried whole milk and distilled water.

‡ The figures in parentheses refer to the number of rats used in obtaining the average hemoglobin value.

§ Rat died.

‖ The average daily dried spinach intake for the 8 weeks experimental period was 1.20 grams, containing 0.43 mg. of Fe and 0.0061 mg. of Cu.

either 0.50 mg. of iron[3] or 0.20 mg. of copper[3] were fed *alone*, a temporary rise in hemoglobin content occurred, followed by an apparent flattening of the hemoglobin curve. Mitchell and Miller (4) who fed 0.1, 0.25, and 0.5 mg. of iron *alone* and Krauss (1) who fed 0.05 mg. of copper *alone*, obtained similar results. Only five of our rats survived at the end of the curative period on 0.50 mg. of iron, death resulting presumably from anemia. Two of the three animals fed 0.20 mg. of copper survived. The ability of

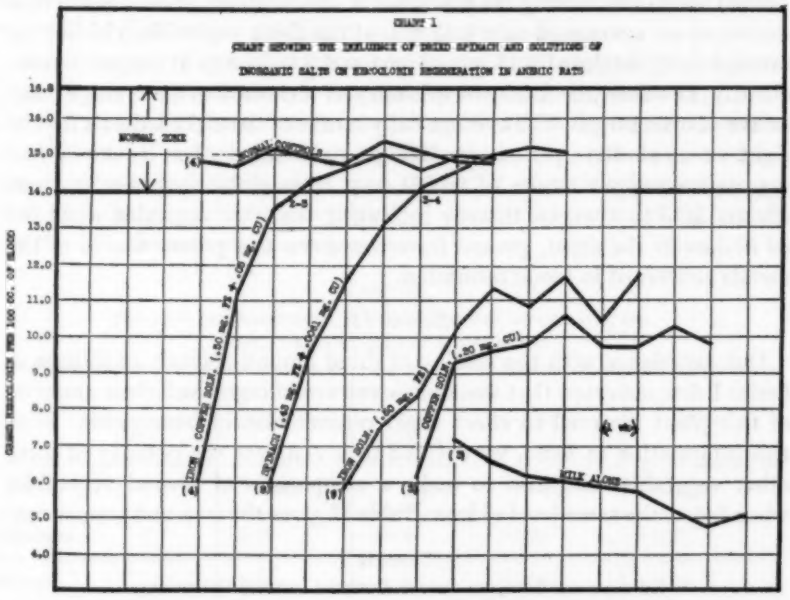

CHART 1. The figures in parentheses at the beginning of each curve refer to the number of animals used in each group. The figures near the top of the curves refer to the time (weeks) required to reach a level of 14.0 gm. hemoglobin per 100 cc. of blood.

either iron *alone* or copper *alone*, to induce partial regeneration of hemoglobin can be ascribed undoubtedly to the presence of small amounts of iron and copper in the milk. When both iron and copper were fed together to the extent of 0.50 mg. of iron and 0.05 mg. of copper, hemoglobin regeneration was speedily effected in 2 to 3 weeks, thereby again confirming the marked supplementary action of copper.

[3] The iron ($FeCl_3$) solution used in these tests was freed from copper by means of H_2S precipitation and, on analysis, was found to introduce only negligible amounts of copper as a contaminant, i.e., 0.000035 mg. of copper daily. Likewise, the copper ($CuSO_4$) solution was found to contain only minute traces of iron.

Before planning an extensive experimental series in which various dried vegetables were to be fed, it was first desirable to ascertain whether anemic rats would consume such dried plant material and also whether the amounts consumed would insure a sufficient intake of iron so that a comparison of the effectiveness of different vegetables could be made possible. Accordingly, dried ground spinach was fed *ad libitum* to a group of eight rats. The iron and copper content of the spinach are given in Table II. Table I shows that during the first week of the curative period, the animals consumed an average of only 0.51 gm. of the dried vegetable, yielding an average daily intake of 0.18 mg. of iron and 0.0026 mg. of copper. Subsequently, however, the intake of spinach was increased to an average daily intake of over 1.0 gm. The average daily intake of the eight animals for the eight weeks curative period was 1.20 gm., yielding an iron intake of 0.43 mg. and a copper intake of 0.0061 mg. Hemoglobin regeneration was effected in 3 to 4 weeks, thereby indicating that this vegetable when fed *ad libitum* in the dried, ground form can serve as a potent source of the metals concerned in blood formation.

Series II

Our experience with the feeding of dried ground spinach *ad libitum* in Series I demonstrated that the anemic rat would ingest sufficient amounts of this plant material to effect rapid regeneration of hemoglobin. With this information at hand, we decided to investigate the potency of some other vegetables and also to make a comparison of several vegetables when fed at the same level of iron. Table II gives the iron and copper con-

TABLE II

SHOWING THE IRON AND COPPER CONTENT OF SOME VEGETABLES

Vegetable	Dry matter (%)	Dry Basis (p.p.m.)		Fresh basis (p.p.m.)	
		Iron	Copper	Iron	Copper
Asparagus	6.7	253	11.0	17.0	0.74
Broccoli	10.5	200	2.0	21.0	0.21
Lettuce	6.5	364	5.3	23.7	0.35
Spinach	9.1	356	5.1	32.4	0.46
Turnip greens	11.0	300	12.5	33.0	1.38
Tomato	5.0	150	14.2	7.5	0.71

tent of the six vegetables used in this investigation, while Table III shows the amounts of iron and copper contained in the daily vegetable supplements.

The vegetables fed were grown in various localities of South Carolina. In our laboratory, Remington and Shiver (15) in a study of eighteen of the more commonly used vegetables grown in South Carolina, found considerable variaton in the iron, copper and manganese content of each vegetable. It is to be emphasized, therefore, that standard values for iron, copper or manganese content cannot be assigned to such plant materials. The samples used in the present investigation represent vegetables having an iron and copper content close to the lower limit of the range in values reported by Remington and Shiver for these vegetables.

As indicated in Table III, a lettuce and tomato mixture was also fed. This mixture was prepared in such proportions that each vegetable furnished one-half of the total amount of iron present in the daily supplement. We were interested in feeding this mixture for the reason that this combination of vegetables is quite often used as a salad. Since turnip greens represent a rather favorite salad in the south, we were interested in determining the effectiveness of this vegetable.

TABLE III

SHOWING THE AMOUNTS OF IRON AND COPPER CONTAINED IN THE DAILY DRIED VEGETABLE SUPPLEMENTS

Vegetable		Amount fed		Equivalent on a fresh basis		Iron		Copper	
		gm.		gm.		mg.		mg.	
Asparagus		0.79		11.8		0.200		0.0087	
Broccoli		1.00		9.5		0.200		0.0020	
Lettuce		0.55		8.5		0.200		0.0029	
Lettuce and tomato mixture	Lettuce	0.28	0.95	4.4	17.8	0.100	0.200	0.0015	0.0110
	Tomato	0.67		13.4		0.100		0.0095	
Turnip greens		1.43		15.7		0.425		0.0179	
Spinach		0.56		6.1		0.200		0.0029	

As indicated in Table III, asparagus, broccoli, lettuce, lettuce and tomato mixture and spinach were all fed at a level furnishing 0.20 mg. of iron daily but different amounts of copper. This low level of iron was chosen instead of a higher level for the reason that we wished to deal with

TABLE IV

AVERAGE WEEKLY HEMOGLOBIN VALUES* AT TIME SUPPLEMENT WAS ADDED AND AT INTERVALS THEREAFTER, OF ANIMALS RECEIVING IRON AND COPPER IN THE FORM OF DRIED VEGETABLES OR SOLUTIONS OF INORGANIC SALTS

	Negative controls on milk	Lettuce and tomato mixture (0.95 gm.)	Asparagus (0.79 gm.)	Lettuce (0.55 gm.)	Spinach (0.56 gm.)	Broccoli (1.00 gm.)	Turnip greens (1.43 gm.)	Iron solution	Iron plus copper solution
Fe in supplement (mg.)	—	0.20	0.20	0.20	0.20	0.20	0.425	0.25	0.25
Cu in supplement (mg.)	—	0.0110	0.0087	0.0029	0.0029	0.0020	0.0179	0.00	0.0170
At time† of addition	5.0 (6)‡	4.4 (5)	4.5 (6)	4.6 (12)	4.3 (13)	4.8 (6)	4.8 (4)	4.2 (6)	4.8 (3)
				After addition (weeks)					
1	5.1 (6)	7.6 (5)	8.2 (6)	7.9 (12)	7.0 (13)	6.1 (6)	7.8 (4)	5.6 (6)	7.8 (3)
2	4.7 (6)	9.2 (5)	10.5 (6)	9.1 (12)	9.0 (13)	7.5 (6)	9.4 (4)	7.3 (6)	10.1 (3)
3	4.7 (6)	12.1 (5)	11.9 (6)	11.8 (12)	11.4 (13)	9.7 (6)	13.5 (4)	8.4 (6)	13.0 (3)
4	4.9 (6)	13.2 (5)	12.8 (6)	12.3 (12)	12.6 (13)	10.1 (6)	14.2 (4)	9.1 (6)	13.6 (3)
5	4.7 (6)	14.0 (5)	13.4 (6)	13.0 (12)	12.8 (13)	11.4 (6)	14.8 (4)	10.2 (6)	14.3 (3)
6	4.6 (6)	14.7 (5)	14.2 (6)	13.6 (12)	13.5 (13)	13.0 (6)	15.1 (4)	11.3 (6)	14.9 (3)
7	4.7 (5)§	15.5 (5)	14.6 (6)	14.3 (12)	14.4 (13)	13.7 (6)	15.4 (4)	11.7 (6)	15.3 (3)
8	4.6 (5)	15.5 (5)	15.1 (6)	15.3 (12)	15.5 (13)	15.1 (6)	15.6 (4)	11.8 (6)	16.2 (3)
Time (weeks) to reach 14.0 gm. hemoglobin	—	4–5	5–6	6–7	6–7	7–8	3–4	—	4–5

* Expressed as grams of hemoglobin per 100 cc. of blood.

† After 4 to 6 weeks of preliminary feeding on dried whole milk and distilled water.

‡ The figures in parentheses refer to the number of rats used in obtaining the average hemoglobin value.

§ Rat died.

a relatively slow rate of hemoglobin regeneration thereby allowing differences in the rate of regeneration of the different vegetables to appear. Mitchell and Miller (4) recommend a level of 0.25 mg. of iron when comparisons are to be made. Turnip greens were fed at a level yielding daily 0.425 mg. of iron and 0.0179 mg. of copper in order to ascertain whether this vegetable would bring about blood formation as rapidly as the spinach did in Series I. Table IV and Chart 2 show the results obtained in this

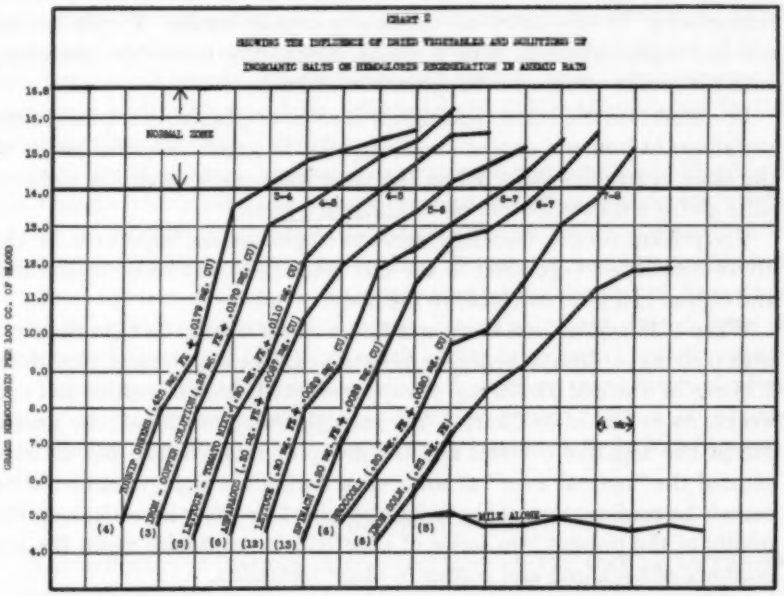
CHART 2
SHOWING THE INFLUENCE OF DRIED VEGETABLES AND SOLUTIONS OF
INORGANIC SALTS ON HEMOGLOBIN REGENERATION IN ANEMIC RATS
GRAMS HEMOGLOBIN PER 100 CC. OF BLOOD
NORMAL ZONE
MILK ALONE
(4 wk.)

CHART 2. The figures in parentheses at the beginning of each curve refer to the number of animals used in each group. The figures near the top of the curves refer to the time (weeks) required to reach a level of 14.0 gm. hemoglobin per 100 cc. of blood.

series. As indicated in the table, beside the vegetable-fed rats, three other groups receiving milk, 0.25 mg. of iron *alone* and 0.25 mg. of iron plus 0.0170 mg. of copper respectively, were included.

In this series, the average hemoglobin content of the rats' blood was reduced to approximately 4.5 gm. per 100 cc. of blood, i.e., 30 per cent normal, before supplements were fed. This value was 10 per cent lower than that in Series I and therefore represents a more marked anemia. The importance of a low initial hemoglobin in studies on nutritional anemia has been pointed out by Mitchell and Miller (4).

From a study of Table IV and Chart 2, it is evident that all the vegetables studied exhibited the capacity to regenerate hemoglobin in animals suffering from nutritional anemia. In comparing the different vegetables, the data show that the vegetables brought about hemoglobin regeneration at rates depending on the copper content. Thus, lettuce and tomato mixture, asparagus, lettuce, spinach, and broccoli—all fed daily at a level affording 0.20 mg. of iron but different amounts of copper—permitted hemoglobin regeneration in 4 to 5, 5 to 6, 6 to 7, 6 to 7, and 7 to 8 weeks, respectively, in the order of decreasing copper intake. Turnip greens effected regeneration in 3 to 4 weeks, comparing favorably, therefore, with the results obtained with spinach in Series I.

In connection with these findings, it is to be emphasized that due to the variations in iron and copper known to exist in vegetables, another set of the same vegetables when fed on the same basis would probably yield results giving a different order of anti-anemic potency.

The present results, therefore, serve to emphasize the importance of the above-mentioned vegetables in the diet since they furnish both iron and and copper in a form available to the body.

When 0.25 mg. of iron *alone* was fed, a result similar to that obtained with 0.50 mg. of iron in Series I was obtained. The supplement furnishing 0.25 mg. of iron and 0.0170 mg. of copper effected blood formation in 4 to 5 weeks. As indicated in Chart 2, the hemoglobin values for all the groups except the negative controls and the group of rats fed 0.25 mg. of iron, entered the "normal zone" at some time during the eight-weeks curative period. In confirmation of the results obtained by other investigators, the results of the present two series of experiments emphasize again the importance of both iron and copper in blood formation.

Discussion

The results obtained in the feeding of dried vegetables serve to demonstrate that such foods are of great importance in the maintenance of normal blood formation. It is apparent that the vegetables studied furnish both iron and copper in a form available to the body for the formation of hemoglobin. The extent to which vegetables contribute to the iron and copper content of sixteen different menus given by Rose (18) for children, adolescents, men and women, has been studied by Hodges and Peterson (19). These workers found that vegetables and cereals are the chief contributors of iron to the diet and supply about equal proportions of this element, i e., about 21 to 24 per cent of the total iron. In respect to copper, vegetables with an average contribution of 19 per cent rank second to cereals supplying 35 per cent.

In confirmation of the results of Waddell and co-workers (16), the results of the present experiments bring out the importance of the small amount of copper which can render iron effective. In our study, broccoli furnished the lowest amount of copper, i.e., 0.0020 mg. daily, which, in conjunction with 0.20 mg. of iron, effected blood regeneration in 7 to 8 weeks. The Wisconsin investigators (16), in their studies involving solutions of inorganic salts, found as little as 0.0025 mg. of copper to possess the ability to induce hemoglobin formation in anemic rats. In our experiments, the group of rats fed the highest amount of copper experienced the most rapid blood regeneration. Thus, in the vegetable-fed groups receiving 0.20 mg. of iron, the lettuce and tomato combination which furnished the largest amount of copper, i.e., 0.0110 mg. daily, effected hemoglobin regeneration in the shortest time, i.e., 4 to 5 weeks.

In Series II, whereas lettuce alone permitted regeneration of blood in 6 to 7 weeks, the lettuce and tomato combination effected recovery in only 4 to 5 weeks. This difference in the rate of regeneration can be ascribed to the higher copper content of the tomato. Remington and Shiver (15) in a study of eighteen of the more commonly used vegetables, found the tomato to contain the largest amount of copper. Since the tomato is much lower in iron than lettuce, and vice-versa much higher in copper, it would appear that these two vegetables supplement each other to make a combination much more effective than either vegetable alone. From a nutritional standpoint, therefore, a lettuce and tomato salad is to be highly recommended.

Summary

The influence of various dried vegetables in the cure of nutritional anemia was studied.

Dried spinach when fed *ad libitum* and yielding an average daily intake of 0.43 mg. of iron and 0.0061 mg. of copper, effected hemoglobin regeneration in 3 to 4 weeks.

Lettuce plus tomato mixture, asparagus, lettuce, spinach, and broccoli—all fed at a level affording 0.20 mg. of iron but different amounts of copper—permitted hemoglobin regeneration in 4 to 5, 5 to 6, 6 to 7, 6 to 7, and 7 to 8 weeks, respectively, in the order of decreasing copper intake. Since variations in the iron and copper content of these vegetables are known to exist, it is to be borne in mind that a different order of anti-anemic potency would probably result from another set of the same vegetables. Turnip greens fed at a level furnishing 0.425 mg. of iron and 0.0179 mg. of copper brought about rapid regeneration in 3 to 4 weeks.

When fed at the same level of iron, a lettuce and tomato combination was found more effective than lettuce alone.

The above vegetables are, therefore, important sources of the minerals concerned in normal blood formation.

Iron *alone* or copper *alone*, when fed in the form of inorganic salt solutions, permitted only partial blood regeneration, whereas solutions containing both iron and copper effected rapid recovery of hemoglobin.

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THE RELATIVE QUANTITIES OF THE HEAT-STABLE AND HEAT-LABILE FRACTIONS OF VITAMIN B IN RAW AND EVAPORATED MILK*

By

LEO T. SAMUELS AND FRED. C. KOCH

(From the Department of Physiological Chemistry and Pharmacology of the University of Chicago)

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INTRODUCTION

DURING the last five years the distinction of at least two factors in vitamin B has been well established (10, 12, 29, 5). As early as 1920 Emmett and Luros (10) showed that one factor could be destroyed by autoclaving at 120° C. for two hours or more. Funk and Dubin (12) showed differences in adsorbability on fuller's earth, and Chick and Roscoe (5) showed differences in solubility in alcohol. The heat-labile substance, more soluble in alcohol, cured polyneuritis in pigeons. The heat-stable fraction was thought by Goldberger (13) to relieve pellagra.

Of particular interest is the evidence which Evans (11), Sure (30), Macy (21), Hartwell (15), Daniels (6), and others have given of the dependence of the mother's milk upon the vitamin B content of her diet, and of a large dissipation of the vitamin in her body, with consequent interference with the adequacy of her milk normally to support the young. Macy (21, 24) has shown by direct growth experiments that human milk and cow's milk are both relatively low in this vitamin, and she, as well as Hunt and Krauss (17), Daniels (6, 7), and Hartwell (15) give evidence that the antineuritic fraction is particularly low in cow's milk. Bloxsom (2) showed that the addition of yeast to infant dietaries increased the rate of growth 60 to 100 per cent. It then may well be that vitamin B is below the optimum level in many infant dietaries.

With the increasing number of infants who are artificially fed and the greater use of evaporated milk for this purpose, as advocated by Marriott (22) and Brenneman (4), together with the interest aroused in the use of milk for the treatment of pellagra, the relative amounts of the factors of the B complex in normal fresh cow's milk and evaporated milk become an important problem. This has been attacked by a number of investigators with confusing results, due to differences in method and in the degree of accuracy obtained. Dutcher, Francis and Combs (9), using direct feed-

* This work was done during the tenure of a fellowship from the Evaporated Milk Association.

ing experiments and preparing the evaporated milks in the laboratory could find no difference between raw and heat-treated milks. However, the amounts of milk fed were low and the experiments were discontinued at a time when differences seemed to be appearing in the growth curves. Johnson and Norton (18) reached the same conclusions in regard to the two types of milk, as did Daniels and Loughlin (8) in some early studies but recently Hartwell (15), and Daniels and co-workers (6, 7), using Hartwell's lactation method, have found great differences. Sure (33) has criticized this work on the basis of failure to deplete the reserves of the mothers, and inadequacy of the basal ration in minerals. It was considered worth while therefore to attempt a quantitative comparison in raw and evaporated milk of the factors of the B complex which could be clearly distinguished.

Experimental

The experimental work can conveniently be divided into three parts:

A. The separation of the two factors by fractionation of the milk solids and confirmation that the limiting factor is the heat-labile fraction.

B. Quantitative estimation of the relative amounts of the heat-labile fraction in raw and evaporated milks.

C. Quantitative estimation of the heat-stable fraction in raw and evaporated milks.

The rats used for the growth experiments were from stock litters weaned on the twenty-first day at a weight of 40 to 50 gm. At that time they were transferred to cages with one-half inch mesh wire false bottoms, which were thoroughly cleaned and sterilized once a week. They then received the basal ration plus water *ad libitum* until they ceased to gain weight, when they were divided and fed the substances to be tested in addition. Where comparisons were to be made, care was taken to distribute comparable litter mates of the same sex among the various groups. All substances fed in addition to the basal ration occupied separate containers. Thus, consumption could be accurately determined. If the substances were fed in a moist powdered, or liquid form there was little if any loss.

The basal ration consisted of:

Casein, purified	20 parts
Starch, Harris, B-free	64 "
Butterfat, purified	8 "
Agar agar, Merck	4 "
Salt mixture, McCollum	4 "
Cod liver oil, Squibb	2 "

In our later experiments one drop of cold liver oil was given with the test substance to insure adequate intake of the fat-soluble vitamins.

The casein was prepared from commercial casein by dissolving in dilute ammonium hydroxide and precipitating with dilute acetic acid according to a modification of the method of Bosworth and Van Slyke (3). The solution and precipitation were repeated four times. The precipitated casein was then held for four days in 0.01 per cent acetic acid, the acid being changed at least once a day. Next the casein was treated for 24-hour periods with three portions of ethyl alcohol, and finally extracted with two portions of ether. The resultant white, friable, tasteless product was dried in a current of warm air and ground to a fine powder.

The butterfat was purified by melting creamery butter on the water bath until the fat layer had thoroughly separated, and then filtering this layer through filter paper in a hot water funnel.

We used autoclaved yeast as the source of the heat-stable fraction and an antineuritic extract prepared according to the method of Kinnersley and Peters (19, 20) for the heat-labile fraction in connection with the milk studies.

The yeast was prepared by drying Fleischmann's starch-free yeast in a current of warm air after crumbling the cakes. The dry yeast was then ground to a powder. The powder was spread in thin layers in tin pans and autoclaved at a steam pressure of 15 lbs. for four hours. It was then dried again in warm air and ground to a powder. When this was fed in quantities of 1.0 gm. a day as an addition to the diet, rats which had come to a standstill on the basal ration gained weight for a brief period and then rapidly declined, death occurring in 23 to 26 days. Toward the end of their days all showed convulsions. Typical curves are shown in Chart 3.

For the antineuritic extract the Kinnersley and Peters procedure was followed in most cases as far as the N/10 hydrochloric acid extract of the norite charcoal. It was preserved by addition of alcohol to give 15 to 20 per cent concentration. To avoid the use of mercury the modification (20) was used in which a norite adsorption at pH 2.5 was substituted for the mercuric sulfate precipitation, except in two extracts, where the regular procedure was followed. With this extract alone as the source of this vitamin, the rats failed to gain but maintained a level of weight for three to four weeks after which they gradually declined. See also Chart 3.

When the autoclaved yeast was fed with the antineuritic extract, however, the rats grew normally to adult size. The two products must then supply all of the vitamin B complex.

A. Separation of the two factors by fractionation of the milk solids and confirmation that the limiting factor is the heat-labile antineuritic fraction:

In the first experiment an attempt was made to separate the two water-

soluble B fractions on the basis of their difference in solubility in alcohol, as shown by Salmon (26), Chick and Roscoe (5), and Sherman (27). Sixteen quarts of certified milk were mixed in a large container after the cream had been drawn off. This milk was then extracted three times with equal volumes of ether previously washed with water. Five liters of the ether-extracted milk were placed in a large flask and aërated until the ether was completely removed. This was then filled into 150 cc. Erlenmeyer flasks and stored at a temperature of −4 deg. C. This served as a control on the ether extraction.

After the lipoids had been removed so as not to interfere with the extraction, the balance of the milk was placed in shallow pans and evaporated to dryness in a current of warm air, ground to a powder and 13/16 of the total milk solids extracted with 85 per cent alcohol. The extract supposedly contained the heat-labile fraction and the residue contained the heat-stable part. As a source of fresh milk for comparison, unpasteurized milk was used.

Chart 1 illustrates the results obtained in this experiment. The first curve shows that the rats made some growth when the extract and residue were combined in amounts equivalent to 15 cc. of the ether-extracted milk but soon leveled off. When 15 cc. of the ether-extracted milk were fed instead, the rats again started to grow, although at a sub-normal rate. There was obviously a loss between the ether extraction and the final alcohol extract and residue. Was this due to the drying or the alcohol extraction? The two curves at the bottom of the chart show that the dried, ether-extracted-milk powder was just as efficient a source of the complex as the original, fresh certified milk. No destruction had occurred in drying; the loss took place during the alcohol extraction. This also showed that 15 cc. of milk could not adequately supply the two factors.

The second and third curves on the chart show that the extraction, however, effected a distinct separation. The second curve duplicates the control curves with autoclaved yeast. The rats did not develop polyneuritis as early as is usual with the rats on the autoclaved yeast, however, which would seem to indicate a slight trace of the antineuritic substance still present. The third curve, in which the extract alone was fed in addition to the basal, is a typical curve as given in the presence of the heat-labile fraction only. Evidently the alcohol extracted practically all of the heat-labile, antineuritic substance without extracting much of the heat-stable fraction. This gives positive evidence of the presence of the two fractions in milk, but the loss in the process prevents its use for quantitative work.

The second series of studies was devoted to determining which was the

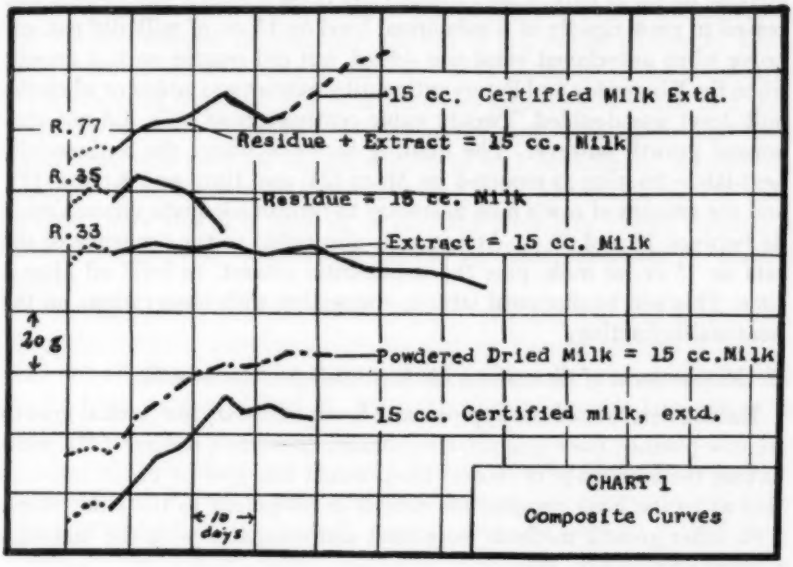


CHART 1
Composite Curves

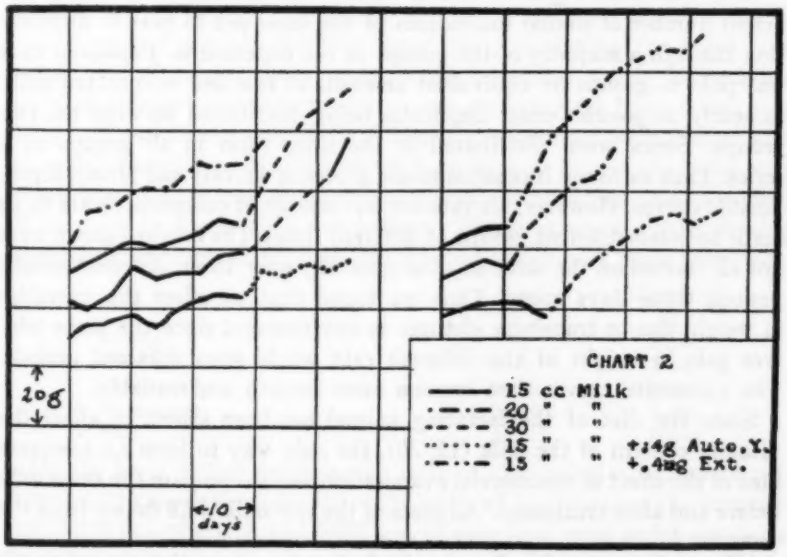


CHART 2

limiting factor in milk. Chart 2 illustrates these findings. Rats which had ceased to grow rapidly at a subnormal level on 15 cc. of milk did not improve when autoclaved yeast was added, but did resume normal growth when the Kinnersley and Peters antineuritic extract was added or when the milk level was doubled. Twenty cubic centimeters of milk did not give normal growth however. The limiting factor is, then, the antineuritic, heat-labile fraction as reported by Macy (24) and Hunt and Krauss (17), and the amount of cow's milk necessary to furnish adequate vitamin must lie between 20 and 30 cc. Attention is also called to the tendency of the rats on 15 cc. of milk, plus the antineuritic extract, to level off after a time. This will be discussed later in connection with observations on the heat-stable fraction.

B. Measurement of the antineuritic heat-labile fraction in milk.

Having determined the approximate levels necessary for normal growth we now planned more quantitative studies. Sherman's method (28), while having the advantage of shorter time, would not give us direct information as to the level required for normal development to the adult stage. Two other growth methods were used and compared with the lactation method of Sure (31, 32).

In all quanitative growth studies litters were selected which had a sufficient number of similar individuals of the same sex to permit distribution through a majority of the groups in the experiment. Particular care was paid to groups on equivalent amounts of raw and evaporated milk, as nearly as possible exact duplicates being distributed between the two groups. Sexes were distributed in the same ratio in all groups of a series. Thus we found it possible to use groups of six rats and obtain reproducible curves. However, six rats are not enough, if comparisons are to be made between different groups at different times. The rats in a group were not all started on the same day but generally over three different weight periods three days apart. Thus we found that we offset the variables in weight due to transitory changes in environment since the same relative gain in weight of the different rats would span different periods. The composite curves then become more smooth and readable.

Since the diet of the lactating animal has been shown to affect the vitamin content of the milk (15, 30), the only way to form an adequate idea of the effect of commercial evaporation was to compare the same milk before and after treatment.[1] Aliquots of the raw milk were drawn from the

[1] Through the courtesy of the Carnation Milk Products Co. we were able to obtain these milk samples under ideal conditions.

thoroughly stirred receiving tanks, stored in ice, thoroughly mixed, sealed in tins and shipped immediately, packed in ice, to the university where they were stored at a temperature of −4° C. Samples of the evaporated milk produced from this raw milk were also shipped and stored at a temperature of 5° C. These two samples of the same milk before and after treatment were used for the main series in each experiment. As controls, samples of certified milk and evaporated milk, purchased on the open market, were run at a level of 15 cc. This level was chosen because it shows the greatest variation in growth per unit difference in vitamin intake.

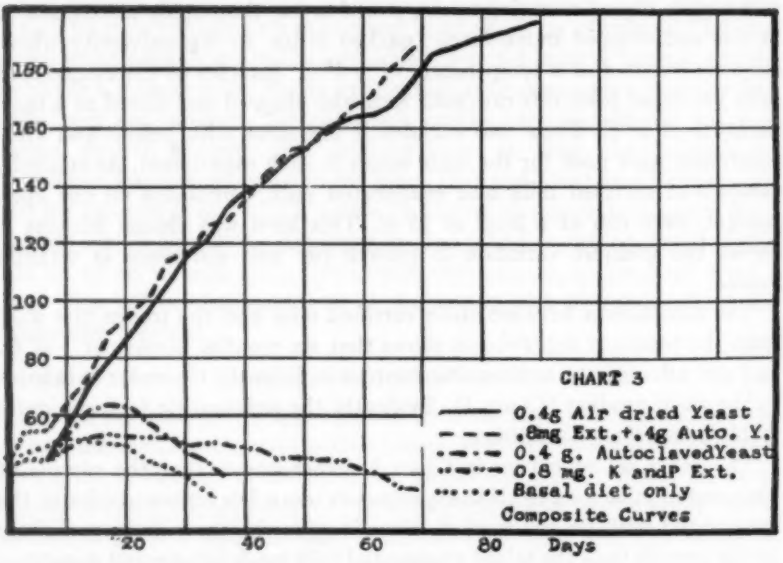
The comparison between fresh certified milk and the frozen raw milk from the company supplying it shows that six months' storage at −4° C. had not affected the antineuritic content sufficiently to render it inferior to the fresh product (Chart 4). Evidently the antineuritic factor is quite stable under these conditions.

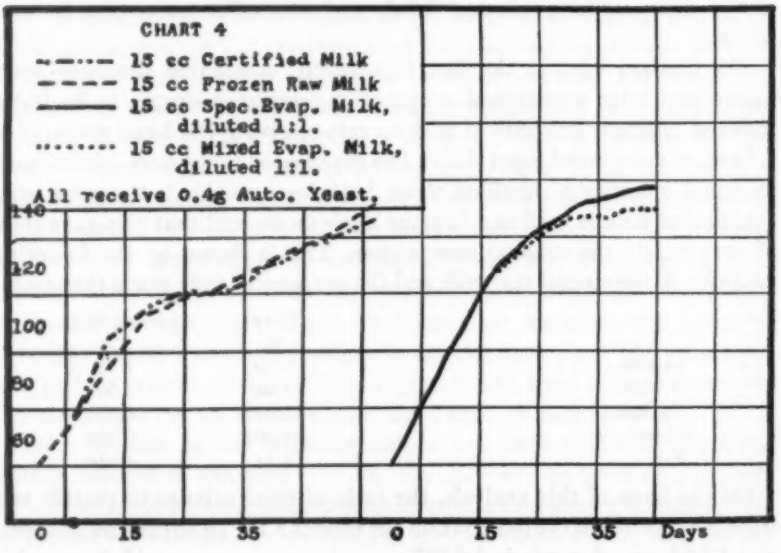
The other set of curves in Chart 4 demonstrates that the sample of evaporated milk used in these experiments was a fair representative of the evaporated milk on the market, since it gave the same or very slightly better growth than the mixed evaporated milk made up of equal quantities of two milks under producers' labels and two milks under distributors' labels.

The method used in the first experiment, comparing the same milk before and after commerical evaporation, consisted simply in feeding a series of different amounts of milk to rats receiving the basal ration plus 0.5 gm. of autoclaved yeast daily. The evaporated milks were diluted with an equal quantity of distilled water both because this is the usual way evaporated milk is used and because analysis showed that this gave milk of very nearly the original composition. This is shown by the following analyses on the special raw milk and the evaporated milk made therefrom:

	Raw	Dilute Evap.
	%	%
Lactose	5.10	5.05
Protein	3.48	3.50
Calcium	0.129	0.130
Phosphorus	0.093	0.100
Fat	3.81	Undet.

On the basis of this analysis, the ratio of total calories to protein calories is 4.63 while in the basal ration the ratio is 5.14. To adjust the composition 0.018 gm. of starch and 0.007 gm. of agar agar were added per cubic centimeter of milk. The results are shown in the composite curves in Chart 5. The amounts of evaporated milk noted on all the charts are





amounts of evaporated milk diluted 1:1 with water. The curves for the raw milk in this series are slightly above those for the same quantity of

diluted evaporated milk until the quantities of both are high enough to give normal growth. Twenty-five cubic centimeters of raw milk have given better growth than 25 cc. of evaporated milk, or 20 cc. of either, but no better than 30 cc. of evaporated. These both give about the same growth as the normal controls, so that the ratio of the antineuritic vitamin must be inversely as 25 cc. raw to 30 cc. dilute evaporated. That the method is sensitive to the differences we are measuring, is shown by the rapid break when the amounts in two groups were lowered and the way in which one group again crosses the lower levels when returned to the higher quantities.

TABLE I

Group	Milk fed	Antineuritic extract cc.	Frac.* normal	Milk** equivalent	Av. Wt. 50 days on diet.
90	None	0.0	0	—	dead
91	15 cc. dil. evap.	0.0	0	15 cc. dil. evap.	122.5
92	" " "	0.2	⅕	22.5 " " "	155.4
93	" " "	0.4	⅖	30 " " "	186.7
94	" " "	0.5	½	33.75 " " "	191.0
97	15 cc. raw	0.0	0	15 cc. raw	134.2
98	" "	0.2	⅕	21 " "	174.8
99	" "	0.3	⅓	24 " "	185.9
100	" "	0.4	⅖	27 " "	188.0
103	15 cc. certified	0.0	0	15 —	132.0
96	None	0.8	1	—	187.5
102	Dried yeast 0.5 g.				193.0

* This column gives the fraction of the standard dose of the antineuritic fraction which was fed.

** This column gives the amount of milk to which this diet is equivalent on the basis of Group 99 as the minimum normal for raw milk and Group 93 as the minimum normal for dilute evaporated milk. Thus, since 15 cc. of raw milk are equivalent to ⅗ of the normal, ⅕ of the normal dose of antineuritic extract must equal 6 cc. of milk, and the diet of Group 98 must be equivalent to 15+6 cc. = 21 cc.

The above method, while giving fair results, is open to criticism since while one may adjust the milks to resemble the basal ration on a gross basis, it is still true that with varying quantities of milk one also varies the amounts of lactalbumin, lactoglobulin, lactose, etc. The best method of measuring the vitamin value of a substance would be one in which only the vitamin itself would be varied. The second method was devised to approach this condition. All groups were fed the same subnormal level of milk, 15 cc. lying in the range of widest weight variation per unit vitamin. To this were then added varying quantities of an accurately standardized anti-

neuritic extract prepared as previously mentioned. Of this preparation 0.858 mg. daily gave normal growth up to at least 200 gm. body weight. Since the highest level of this fed to any milk group was 5/8 of the normal (0.536 mg.), this was the widest variation in intake aside from the basal ration, thus lowering this possible error considerably. Six rats were used in each group, four males and two females. Great care was taken to have uniform general distribution. The results are given in Chart 6 and Table I. The curves show even better results than the previous experiment. According to this series 24 cc. of raw milk are equivalent to 30 cc. of the diluted evaporated milk and will give normal growth. The ratio is then between 4:5 and 5:6. In other words approximately 1/5 of the antineuritic heat-labile fraction is destroyed in the process of commercial evaporation.

In our last series of experiments we used a lactation method, since the experiments of Hartwell and Daniels by Hartwell's method had indicated wider differences between raw and evaporated milks. However, to avoid the errors in Hartwell's diet pointed out by Sure (33), we decided upon a a method very similar to that of the latter workers (32). Females were transferred upon the day of birth of their litters to the vitamin B-free basal ration plus an allowance of 1.0 gm. of autoclaved yeast daily. The litters were reduced to six within three days after birth. Since the young at this age would fall through the false bottoms of our cages, they were placed on pure white crepe paper in the bottom of the cage, but were transferred to the false bottoms as soon as they grew sufficiently. This was

TABLE II
LACTATION EXPERIMENTS WITH EVAPORATED AND RAW MILK

No.	No. in litter	Days on basal	Av. wt. at transfer	Days on milk	No. weaned	Ave. wt. weaned	Condition
40 cc. raw milk:							
362	6	14	12.4	22	0		Squealing fits, convulsions
250	6	16	24.0	16	6	41.5	
337	6	16	20.3	24	1	37	Rats died in severe convulsions
50 cc. raw milk:							
243	6	16	22.4	15	6	42.3	Normal
245	6	16	17.8	17	6	40.7	Normal
339*	6	3	5.4	23	6	39.8	Normal

TABLE II (cont'd)

60 cc. raw milk:							
358	6	15	16.6	18	6	40.8	Normal
70 cc. raw milk:							
254	6	17	24.6	9	6	40.3	Normal
328	6	16	15.9	13	6	39.6	Normal
296†	6	14	19.3	20	6	33.3	2 rats had running fits
25 cc. undiluted evaporated milk:							
293‡	6	15	18.5	17	6	37.0	2 rats had convulsions, very weak
170	6	11	13.6	19	5	40.7	1 rat died in convulsions
312	6	18	19.4	15	0		Screaming fits, convulsions later
30 cc. undiluted evaporated milk:							
244	6	15	20.0	13	6	40.8	Normal
249	6	18	18.2	21	5	40.1	1 rat died, possible convulsions
15	6	18	20.4	17	6	35.6	Normal
350§	5	22	25.8	10	5	39.4	Normal
40 cc. undiluted evaporated milk:							
301	5	17	21.2	13	5	40.8	Normal
359	6	20	19.3	14	6	37.2	2 runts which showed slight signs of conv.
240	6	17	13.4	17	6	42.5	Normal
Controls on Basal diet plus Auto. yeast only:							
346¶	6	34			0		Screaming fits and convulsions
351	5	17			0		Screaming, running fits
30	5	19			0		Screaming fits, convulsions

* Mother did not nurse litter well, so milk was given to her soon after birth. She then began to nurse the young.

† This litter was rather weak at time of transfer. Two of the young did not develop well and showed fits soon after transfer.

‡ Two of the young were smaller than the others and died after weaning.

§ This rat left some milk during part of experimental period.

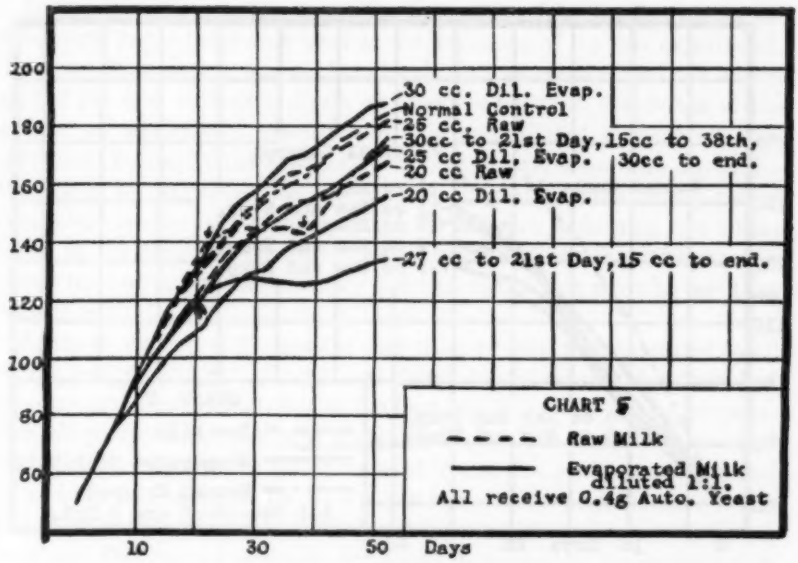
¶ Mother lost weight rapidly and developed severe polyneuritis, but some of young while scrawny did not die, probably because they ate some of their dead mates.

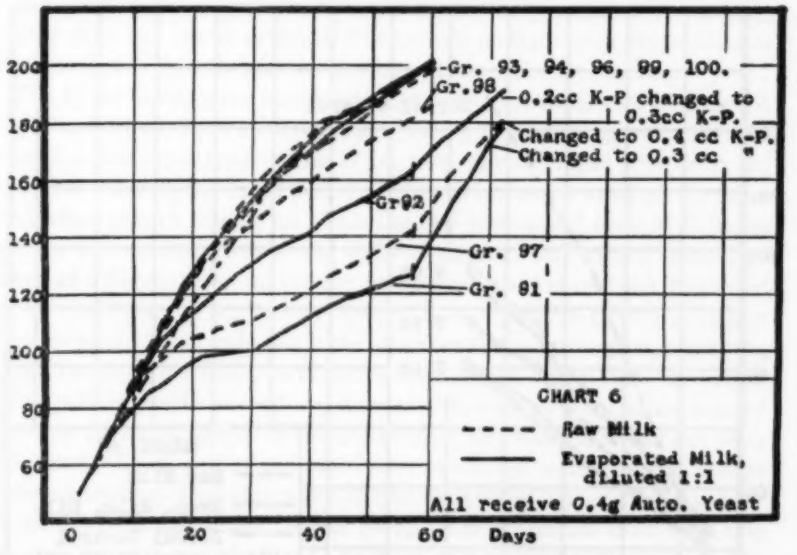
within three to six days. When the weight of the litters became stationary, which occurred generally from the 14th to the 18th day, the material to be tested was fed in a separate container suspended from the top of the cage so that the young could not reach it. Litters were raised thus until they died or became large enough to reach the milk. The results of this experiment are given in Table II. The rats react somewhat variably, but a comparison of the records shows that the group on 50 cc. of raw milk raised most of the litters to an average weight of forty grams. The group receiving 30 cc. undiluted evaporated milk, equivalent to 60 cc. diluted, reacted similarly, but 25 cc. undiluted was too low. The ratio of the two milks with respect to their value for lactation is then of the same order as the ratios found in the growth experiments.

C. Measurement of the heat-stable fraction:

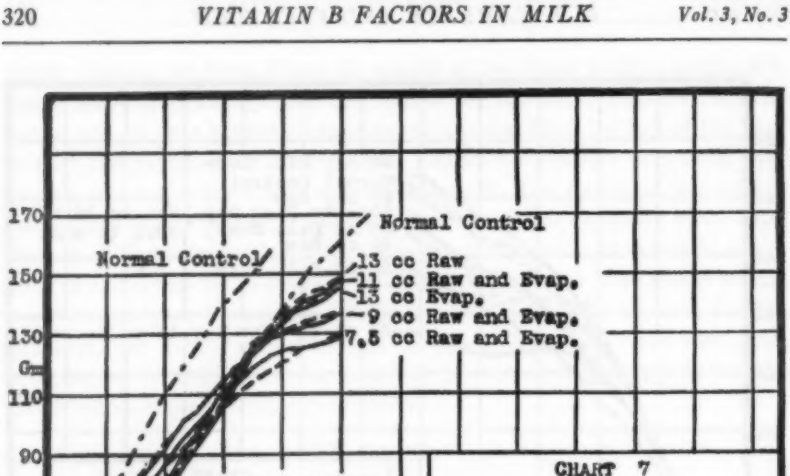
Only one method has been used in this experiment, the method of adding various amounts of milk to a diet containing all constituents known to be essential for the rat, except the heat-stable fraction. The method was similar to that of Aykroyd and Roscoe (1) except that the growth periods were longer, parallel controls were always run, and rats were very carefully balanced. The method of adding a relatively pure extract has not been practicable here since it was found impossible to prepare concentrates from yeast which in small quantities would give normal growth to the adult stage. Concentrates prepared from the various fractions of the Kinnersley and Peters residues varied widely in activity. In fact our results tended to confirm the evidence brought forward by Reader (25), Williams and Waterman (34), Hunt (16), and others, of a multiplicity of these factors. The original experiment consisted simply in the separate feeding of measured quantities of the raw and evaporated milks to carefully balanced groups of rats receiving an otherwise adequate ration made up of the basal diet plus a daily addition of a standardized antineuritic extract. In the second series the rats were even more carefully selected and the milk additions were all made up to the same volume to eliminate possible errors due to variation in fluid intake. The results are particularly significant in regard to the comparison of the two types of milk.

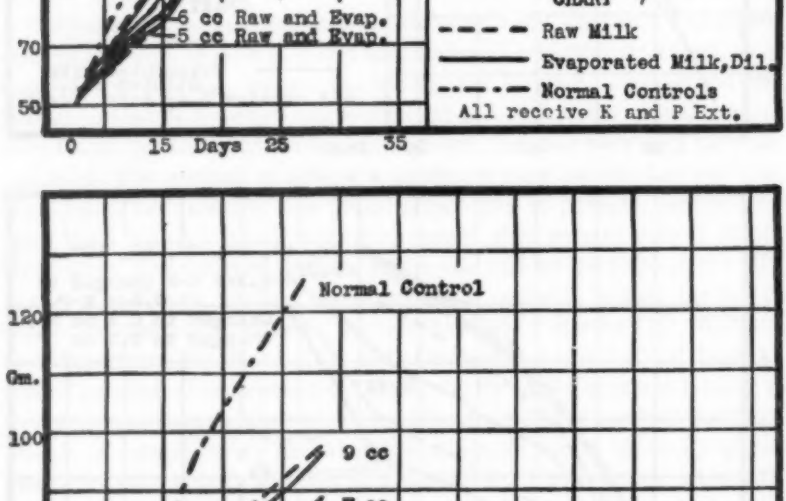
As was previously mentioned, the preliminary experiments indicated that the level of milk necessary to furnish adequate quantities of the heat-stable factor was lower than for the heat-labile factor. Consequently a series of 5, 6, 7.5 and 9 cc. of each milk (raw and 1:1 diluted evaporated) was begun. However, none of these amounts gave normal growth so after 17 days the groups on the two lower levels were increased to 11 and 13 cc. respectively. These quantities gave normal growth for some time but





then they too failed to keep up. Throughout this whole experiment however, as will be noticed in Chart 7, equal quantities of raw and diluted evaporated milk gave almost superimposable curves. It is also interesting to note that the 11 and 13 cc. levels did not separate significantly.


170
150
130
Gm
110
90
70
50
Normal Control
Normal Control
13 cc Raw
11 cc Raw and Evap.
13 cc Evap.
9 cc Raw and Evap.
7.5 cc Raw and Evap.
6 cc Raw and Evap.
5 cc Raw and Evap.
CHART 7
Raw Milk
Evaporated Milk, Dil.
Normal Controls
All receive K and P Ext.
0
15 Days 25
35


120
Gm.
100
80
60
Normal Control
9 cc
7 cc
5 cc
CHART #8
Raw Milk
Evap. Milk, Dil.
Normal Control
All receive KandP Ext.
0
7.5
17.5 Days

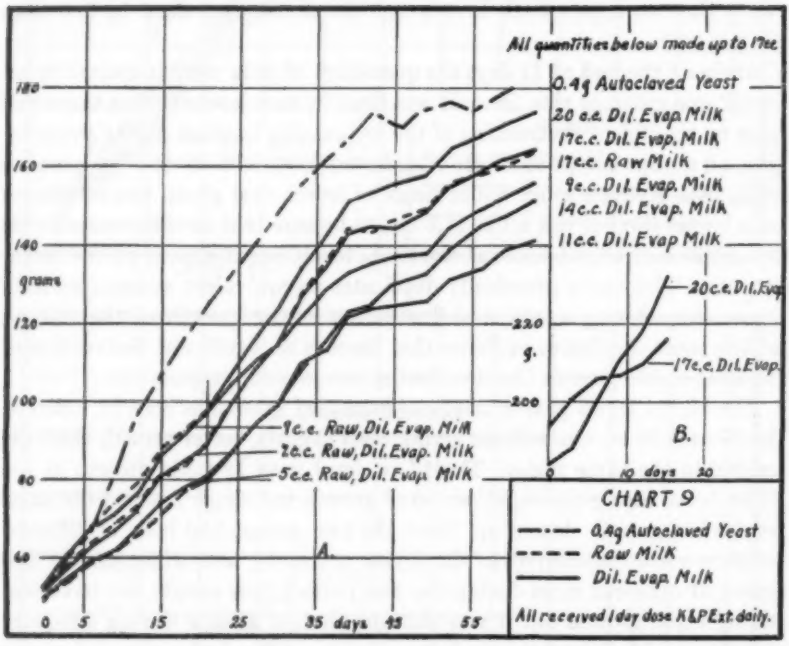
In the second series the quantities first selected for feeding lay in the subnormal range previously used at the beginning of the first experiment. This was done since it was at these levels that we could expect the widest spread per unit variation. Again as shown in Chart 8, the curves of the groups on equivalent quantities of raw and diluted evaporated milk coincided. Evidently the destruction by heat, if any, is less than can be distinguished by careful feeding experiments, since at no point do any pair of equivalent curves vary more than 1/3 the distance separating two intake levels at the time the quantities were changed. Since the difference between the two upper levels is only 2/9 the destruction must be less than ten per cent.

Again at the end of 21 days the quantities of milk were increased to all except one group of rats. Since it was clear by now however that there had been no significant destruction of the heat-stable fraction during evaporation, all groups except one were transferred to various levels of evaporated milk, thus giving a much wider range of levels. One group was continued on a higher level of raw milk, 17.0 cc., to be sure that no differences in the two milks indistinguishable at the lower levels might appear on the larger amounts. This curve practically duplicates its equivalent evaporated milk curve, even during a period of fluctuation in the growth of the colony, adding more conclusive evidence that there is no significant destruction of the heat-stable growth fraction during commercial evaporation.

The higher levels gave immediate increased growth as seen in Chart 9, the 17 and 20 cc. equivalents giving even slightly better growth than the control in the same region. The 17 cc. level gave as rapid growth as the 20 cc. level during the rapid period of growth but never reached the same level when growth slowed up. Since the two groups had been of different weights when transferred to the higher amounts, and consequently had grown at different rates during the first period, they should not have met during rapid growth but it was thought that all groups having adequate supplies would eventually reach the same level, the lower groups growing longer. On the other hand, conclusions could not be drawn from the period during which growth slowed up, since at this time we were unfortunately forced to use a new supply of the antineuritic solution which had not been well standardized, and at the same time to change technicians in charge of the rat feeding. That this was a serious disturbing influence is shown by the erratic behavior of the control group. It is impossible then from this series of curves to say whether 17 or 20 cc. of 1:1 diluted evaporated milk would be sufficient for normal growth and maintenance.

As further evidence on this particular question, two groups of rats previously used for standardization of antineuritic extracts were run on these

two higher levels. These do not have the same quantitative significance since the groups were smaller (3 rats) and were not so well balanced as far as litter mates of exactly the same weight were concerned. All were males. The results, shown in Curve 9B, would indicate that even 17 cc. of milk are not sufficient for optimum growth at these higher levels. The group which originally weighed less gained with sufficiently greater rapidity on the equivalent of 20 cc. of milk so that at the end it exceeded the weight of the group on 17 cc. of milk by more than its original negative difference.



However, it must be noted that this group gave better than our usual normal growth at this period.

The results of this series of experiments then indicate that raw and evaporated milk are equally good sources of the heat-stable factor and that the level of intake necessary for optimum growth lies between 17 and 20 cc. daily for the albino rats.

Discussion

The results in the growth experiments show that milk contains all the water-soluble vitamins necessary for the growth of the rat, but that rela-

tively large volumes must be taken to obtain optimum growth. Commercial evaporation has not seriously affected these substances. The small destruction in the case of the antineuritic fraction is probably in large part due to the process of sterilization in which temperatures above 100° C. are maintained for some time. Undoubtedly it is wise to include other sources of these vitamins in all infant dietaries.

Aside from this main point there are a number of very interesting things to be noticed.

When we began the series of lactation experiments, we considered the possibility of another factor being involved in lactation which might be so closely related to the antineuritic fraction that it might appear in the antineuritic extracts which had been used for curative effects. The symptoms of the young rats when first showing these effects are not the same as those seen in older rats on a polyneuritic diet (7, 15, 30). This might explain the discrepancy between the results of Daniels (7) and our growth experiments. However, our experiments indicated the same ratio between the two. Further than that, the rats which developed symptoms after longer growth often showed typical convulsive paralysis, while the younger rats of similar litters on the same diet showed the squealing running fits. Intermediate conditions occurred also. From this we were led to conclude that we were either dealing with the same substance or one which is destroyed at the same rate by heating.

Our observations of the relation of pellagra as a skin disease to the growth-promoting, heat-stable fraction were by no means clear. Like Sherman (27) we have found symmetrical loss of hair and brown scaling more often on our intermediate cases than on the controls totally lacking in the heat-stable fraction. In the latter we had rough, dull, but usually complete coats of hair, bloody irritated breakdown of the tissue around the nasal openings, chromogenic urine, and intestinal symptoms. Occasionally we have had severe skin lesions in these rats although they have been rare. On the other hand we have had case after case of loss of hair over large symmetrical areas with rats on 0.5–1.0 gm. of autoclaved yeast and growing rapidly. More careful correlative work between skin symptoms and growth must be done. However, no clear cut work can be well done until further purification of this fraction is carried out. The progress being made along this line should justify further study.

Summary

Biological analyses by three different methods have shown that commercial evaporation of cows' milk destroys about one-sixth to one-fifth of the antineuritic heat-labile fraction. All cows' milk seems to be quite low

in this factor however, 25 cc. (3.49 gm. solids) being required per rat per day for optimum growth. Milks do not apparently vary widely in their antineuritic content as purchased on the market. There is no identifiable destruction of the heat-stable, growth-promoting fraction during commercial evaporation. Milk is capable of adequately supplying this fraction for optimum growth. The amount necessary, while lower than that required for the antineuritic factor, is also fairly large, that is 17 to 20 cc. (2.37 to 2.78 gm. solids). The question of the identity of this heat-stable factor with the antipellagra fraction is still unsettled.

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